Combined SN-38 and gefitinib treatment promotes CD44 degradation in head and neck squamous cell carcinoma cells

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Abstract. The aim of the present study was to search for an effective regimen among existing chemotherapies for head and neck squamous cell carcinoma (HNSCC). Among the tested drugs, we focused on combined SN-38, which is the active metabolite produced from irinotecan hydrochloride - a type I DNA topoisomerase inhibitor - after it is metabolized by carboxylesterase in the liver and gefitinib, an EGFR tyrosine kinase inhibitor treatment, based on the ability of this combination to inhibit HNSCC cell growth. Contrary to our expectation, *in vivo*, there was no significant difference in tumor growth suppression between gefitinib-only treatment and gefitinib plus SN-38. However, when tumor measurements were continued after treatment ceased, we found that several tumors showed renewed growth in the gefitinib-only group. The tumors that resumed growing after treatment showed increased CD44 expression compared with tumors from the combined treatment group. Next, we investigated the mechanism whereby SN-38 and gefitinib inhibited CD44 expression *in vitro*. These studies revealed that the combined treatment promoted lysosomal degradation of CD44. The present study revealed that combined gefitinib and SN-38 treatment inhibits CD44 expression by promoting its lysosomal degradation in HNSCC cells. However, it is still unclear whether inhibition of CD44 expression in HNSCC cells can directly suppress tumor regrowth after therapy. Thus, it may be necessary to elucidate the relationship between the effects of these chemotherapeutic agents on CD44 expression and tumor recurrence/metastasis in future studies.

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Introduction

The aim of the present study was to identify an effective chemotherapy regimen that suppresses the local recurrence and metastasis of head and neck squamous cell carcinoma (HNSCC). The main HNSCC treatments include surgery, radiotherapy and chemotherapy; however, many refractory tumors recur and metastasize despite treatment. Moreover, the head and neck contain many organs that play important roles in daily functions such as chewing, swallowing, vocalization and respiration. Damage to these organs from side-effects of surgery, radiotherapy or chemotherapy seriously reduces the quality of life of these HNSCC patients (1,2). The first-line chemotherapy for HNSCC is the platinum-containing drug cisplatin, and in cases of recurrence or metastasis, combination chemotherapy consisting of cisplatin and fluorouracil (5-FU) or cetuximab, which is an epidermal growth factor receptor (EGFR) inhibitor, is used (3,4).

In recent years, it has been appreciated that there is a hierarchy to the cells that constitute cancer tissues; most cancer cells are thought to arise from a small number of cancer stem cells, which are located at the top of this hierarchy (5). Studies have demonstrated that cancer stem cells possess properties similar to the self-replication phenotypes of conventional stem cells. Cancer stem cells also have a high differentiation capacity and are resistant to standard anticancer treatments, such as radiotherapy and chemotherapy (6). Therefore, even though it may appear that all cancer cells have been eradicated by what appears to be curative treatment, in most cases, a small number of cancer stem cells remain. When these cancer stem cells regenerate to reform the primary tumor, it is classified as recurrence, while metastasis is defined as the migration of cancer stem cells to other organs, producing a tumor at these sites (5,7). Currently, there are no treatments to control HNSCC recurrence and metastasis.

Biomarkers of HNSCC cancer stem cells include CD44, aldehyde dehydrogenase (ALDH), CD133, c-Met (a tyrosine kinase receptor for hepatocyte growth factor), and drug efflux transporters such as MDR1 and ABCG2 (8). CD44 is a large cell surface glycoprotein associated with tumor growth and migration (9) and the most well-known cancer stem marker

in HNSCC. CD44 binds growth factors, such as EGFR, and metalloproteinases, such as MMP9, resulting in reduced apoptosis, and the induction of angiogenesis and cancer cell invasion (8,10-12). Our previous study revealed that CD44 inhibits apoptosis by modulating DNA damage responses during the G2/M phase of the cell cycle (13). Clinically, it has been found that CD44-positive cells are associated with poor prognosis, more aggressive tumors and higher recurrence rates after radiation therapy (14). Other studies have reported that the microRNA miR-34a is a novel therapeutic target that negatively regulates CD44-positive cells (15).

The underlying question for the present study is whether there is a more effective combination therapy for HNSCC among clinically approved chemotherapeutic agents. While many researchers have reported that cancer stem cells influence recurrence, metastasis and prognosis, it is unclear how existing chemotherapies influence cancer stem cells. Therefore, in the present study, we searched for effective therapies for HNSCC among conventional chemotherapeutic agents and clarified the effects of these drugs on cancer stem cells.

Materials and methods

Reagents and cell culture. The HNSCC cell lines HSC-2, HSC-3, SAS and Ca9-22 were obtained from the Riken Cell Bank (Ibaraki, Japan). The human immortalized non-tumorigenic keratinocyte cell line, HaCaT was purchased from DKFZ (Heidelberg, Germany). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Life Technologies Japan Ltd., Yokohama, Japan), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere containing 5%.

Evaluation of cell growth and determination of drug synergy. Following *in vitro* drug treatment, the number of viable cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the Cell Proliferation Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The number of viable cells was assessed by measuring the absorbance of formazan crystals at 595 nm with TECAN SpectraFluor Plus XFluor4 software (Tecan Japan Co., Ltd., Kawasaki, Japan). Synergetic drug effects were assessed with the CompuSyn™ program (Biosoft, Ferguson, MO, USA) using a combination index (CI) that is based upon the Chou and Talalay median-effect principle (16). The CompuSyn™ software was used to calculate the dose-effect relationship and quantitate synergism and antagonism in drug combinations from IC_{50}, ED_{50} or LD_{50} data based on Chou's median-effect equation (17). CI was used to identify synergistic, additive and antagonistic drug interaction; CI <1 was considered a synergistic effect.

Mice and in vivo tumor growth analysis. Male BALB/c nude mice, 6- to 8-weeks old, were obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The present study was approved by the Animal Ethics Committee of Meikai University (no. C1201). HSC-2 cells ($3x10^6$) were resuspended in 0.1 ml phosphate-buffered saline (PBS) and subcutaneously injected into the lateroabdominal region of male BALB/c nude mice. Tumor volume was calculated using the formula a x $b^2/2$ where 'a' is the length and 'b' is the width of the tumor diameter. Drug administration was initiated when tumor volumes reached ~400-500 mm^3. SN-38 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and gefitinib (LC Laboratories, Woburn, MA, USA) were dissolved in 0.5% carboxymethylcellulose sodium. SN-38 (10 mg/kg) was administered by intraperitoneal injection, and gefitinib (100 mg/kg) was administered by subcutaneous injection. The SN-38 concentration was based on the study by Guo *et al* (18), and that of gefitinib was based on the study by Stewart *et al* (19). When used as combination therapy, SN-38 (10 mg/kg) and gefitinib (100 mg/kg) were administered by independent injections to the same mouse. For vehicle controls, 0.5% carboxymethylcellulose sodium was administered in equal volumes via the respective routes. Each group contained 5 mice that received drug or vehicle injections 5 days/week for 3 weeks. Tumor size was measured every 3 days and this observation continued for 60 days. There were no significant differences in the body weights of the mice. At the end of this experiment, mice were given 5% isoflurane (Wako Pure Chemical Industries, Ltd., Osaka, Japan) inhalation anesthesia and euthanized by pentobarbital Na (Nakalai Tesuque Co., Kyoto, Japan) overdose (~100 mg/kg) via intraperitoneal administration.

Immunohistochemistry. Mice were sacrificed 21 days after finishing treatment (42 days from the initiation of drug administration), and tumor tissues from the gefitinib-only and combination treatment groups were fixed in 10% formalin buffer, embedded in paraffin, and 5-μm sections were placed on positively charged glass slides. EGFR immunohistochemical staining was performed using the EGFR pharmDX kit (Dako, Glostrup, Denmark) according to the manufacturer's protocol. The primary antibody contained in this kit is ready to use, and the response time was 30 min. CD44 was stained separately using a mouse monoclonal anti-human CD44 phagocytic glycolin-1 antibody (DF1485; Dako) at 1:50 concentration for 30 min, which was applied to tissue sections followed by secondary biotinylated antibody and streptavidin-HRP conjugate complex contained in the kit. After washing with buffer, diaminobenzidine was applied followed by a counterstain with Mayer's hematoxylin.

Isolation of CD44-positive cells. CD44-positive cells were isolated from the HSC-2 cell line using magnetic anti-CD44 immunobeads (MACS; Miltenyi Biotec, Berdish-Gladbach, Germany) according to the manufacturer's instructions. Cells that passed through the MACS magnetic bead system without attaching to the magnetic anti-CD44 immunobeads were designated as CD44-negative cells.

Migration assay. Cell migration analysis was performed using BD Falcon cell culture inserts (Becton-Dickinson and Company, Franklin Lakes, NJ, USA), which allows cells to migrate through 8-μm pores in polyethylene terephthalate membranes. Briefly, $1x10^5$ cells were resuspended in serum-free medium and added to the upper Transwell chamber into a 12-well plate. The lower chamber was filled with serum-free DMEM or DMEM containing 10% FBS. After 24 h, cells remaining on the upper surface of the membrane

were removed with a cell scraper. Cells beneath the membrane were fixed with cold 6.0% (v/v) glutaraldehyde for 30 min and stained with 0.5% (w/v) crystal violet. Cells were counted in 10 high-power microscope fields.

CD44 knockdown by siRNA transfection. Small interfering RNA (siRNA) specifically targeting human CD44 [HCAM siRNA (h): sc-29342] was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HSC-2 cells ($1x10^5$/well) were plated into 6-well plates and transfected with CD44 siRNA for 24 h in antibiotic-free media using siRNA Lipofectamine RNAiMAX and Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. After a 24-h transfection, the cells were used for experimentation.

Immunoblot analysis. Whole-cell extracts were obtained in lysis buffer (10X cell lysis buffer; Cell Signaling Technology, Danvers, MA, USA) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) plus one protease inhibitor cocktail tablet (Complete EDTA-free; Roche Diagnostics). Protein concentration in the lysates was assayed, and equal amounts of protein for each sample were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with the following primary antibodies: mouse monoclonal anti-CD44, rabbit monoclonal anti-cleaved PARP, rabbit polyclonal anti-EGFR, rabbit monoclonal anti-phospho-EGFR (Tyr1068), rabbit monoclonal anti-HER2/ErbB2 (all from Cell Signaling Technology and used at a concentration of 1:1,000), rabbit polyclonal anti-phospho-HER2 (Tyr1248) (1:50,000; Merck KGaA, Darmstadt, Germany) and anti-β-actin (1:10,000; Sigma-Aldrich, St. Louis, MO, USA). Signals were detected using the corresponding peroxidase-conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG; Cell Signaling Technology), and immunoreactive bands were visualized by chemiluminescence (Clarity™ Western ECL substrate; Bio-Rad, Hercules, CA, USA). Membranes and images were developed with a ChemoDoc™ Imaging System (Bio-Rad) or ImageQuant™ LAS500 Imaging System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Flow cytometry. HSC-2 cells were harvested by trypsinization, centrifuged into cell pellets and resuspended in FACS buffer (PBS containing 0.5% bovine serum albumin). The cells were stained for 30 min at 4˚C with FITC-conjugated anti-human CD44 antibody at a concentration of 1:50 or an isotype-matched FITC-conjugated IgG control antibody (both from BD Biosciences, San Jose, CA, USA). Flow cytometry was performed using an EPICS Altra flow cytometer, and data were analyzed using Expo-3 v1.2B software (Beckman Coulter, Brea, CA, USA).

Cell cycle analysis. HSC-2 cells were treated with SN-38, gefitinib or the combination for 24 h at 37˚C. Nuclei were labeled with propidium iodide (BD Biosciences), and the DNA contents of propidium iodide-labeled nuclei were measured via flow cytometry according to the manufacturer's instructions. Data acquisition and analysis were performed using an EC800 flow cytometer with EC800 analysis software (Sony Biotechnology, Tokyo, Japan).

Table I. The half-maximal inhibitory concentration (IC_{50}) of SN-38 and gefitinib for HSC-2 and HaCaT cells.

The half-maximal inhibitory concentration (IC_{50})	HSC-2 (head and neck squamous cell carcinoma cell line)	HaCaT (human skin keratinocyte cell line)
SN-38 (nM)	6.2±0.9	32.5±8.6
Gefitinib (μM)	38.5±18.8	0.63±0.01

Proteasome and lysosome inhibition. The proteasome inhibitor MG132 was purchased from Selleckchem (Houston, TX, USA). HSC-2 cells were pretreated with the inhibitors for 30 min before stimulation with chemotherapeutic agents. The lysosome inhibitor chloroquine was purchased from Tokyo Chemical Industry Co., Ltd. HSC-2 cells were pretreated for 1 h before stimulation with chemotherapeutic agents.

Statistical analysis. All quantitative data are presented as the mean ± standard deviation. Data were evaluated using one-way analysis of variance followed by the Turkey-Kramer post-test. P-values <0.05 were accepted as statistically significant.

Results

Combined SN-38 and gefitinib treatment inhibits HNSCC cell growth in a synergistic manner. First, we screened combinations of existing chemotherapeutic agents to find those that synergistically inhibited HNSCC cell growth. Among them, we focused on SN-38, which is the active metabolite produced from irinotecan hydrochloride - a type I DNA topoisomerase inhibitor -after it is metabolized by carboxylesterase in the liver (20) and gefitinib, which is a specific EGFR tyrosine kinase inhibitor (21). The half-maximal inhibitory concentration (IC_{50}) of SN-38 and gefitinib in the HNSCC cell line HSC-2 were 6.2±0.9 nM and 38.5±18.8 μM, respectively (Table I). We next investigated the cell growth inhibition achieved by combinations at different concentrations: one quarter, one half, IC_{50}, and 2- and 4-fold of the IC_{50}. Combinatorial treatment at each concentration significantly inhibited cell proliferation (Fig. 1A). Furthermore, we investigated whether the combined treatment showed additive or synergistic effects by determining the CI. A CI value <1 indicates a synergistic effect, and CI values of 1 indicate an additive effect. All combined SN-38 and gefitinib treatments (different doses) showed CI values <1, revealing that the inhibitory effects on HNSCC cell proliferation were synergistic for this combination (Fig. 1B and C, and Table II). We also analyzed cleaved PARP levels, a marker of apoptosis, and the data suggested that the combination treatment enhanced HNSCC cell apoptosis (Fig. 1D). These data suggested that the combined SN-38 and gefitinib treatment synergistically inhibited HNSCC cell growth and induced apoptosis.

CD44 is expressed in regrowth tumor tissue. Our *in vitro* data suggested that the combined SN-38 and gefitinib treatment could be effective against HNSCC. Therefore, we attempted to

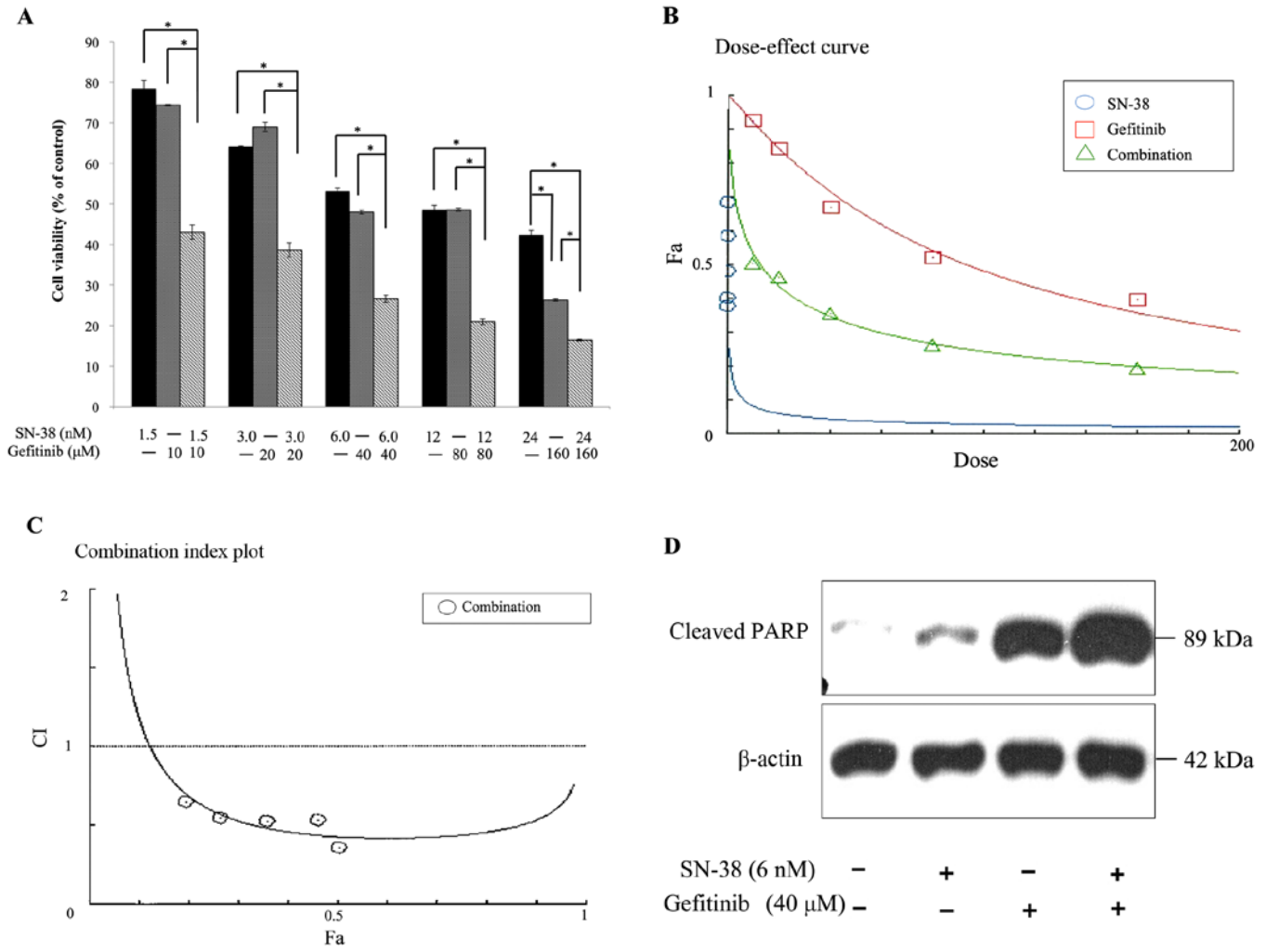

Figure 1. Effect of combined SN-38 and gefitinib treatment on HNSCC cells. From the IC_{50} values of SN-38 and gefitinib in the HNSCC cell line HSC-2, the combinations of 1/4 of the IC_{50}, 1/2 of the IC_{50}, the IC_{50}, 2 times the IC_{50} and 4 times the IC_{50} values were tested for their ability to inhibit cell growth. Error bars indicate standard deviation (SD); $^{*}P<0.01$ (A). The median-effect plot using the Chou and Talalay method was used based on the value of cell proliferation suppression for each concentration combination. Fraction affected (Fa) shows the inhibitory cell ratio and dose concentration on abscissa axes (B). The combination index (CI), which is a quantitative index for the combinatorial effect of the combined treatment, was calculated corresponding to Fa. When CI <1, it was judged to be synergistic (C). The ability to induce apoptosis in HSC-2 cells by combined SN-38 and gefitinib treatment was examined by immunoblotting (D).

Table II. The CI index of SN-38 and gefitinib for HSC-2 cells using the Chou and Talalay method. CI values for actual experimental points.

Total dose (μM)	Fa	CI value
10.0149	0.506	0.35601
20.0298	0.463	0.53007
40.0596	0.358	0.52431
80.1192	0.264	0.54891
160.238	0.195	0.65120

Total dose, the total amount when combining SN-38 and gefitinib from 1/4 of the IC_{50}, 1/2 of the IC_{50}, the IC_{50}, 2 times the IC_{50} and 4 times the IC_{50} values; Fa, the fraction affected in the Chou and Talalay method. CI, combination index.

verify these findings *in vivo* using a xenograft mouse model. Contrary to our expectation, there was no significant difference between gefitinib single treatment and combined gefitinib and SN-38 treatment in this model (Fig. 2A). However, tumors in 3 of 5 mice from the gefitinib single administration group showed renewed growth 6 weeks (42 days) after terminating treatment (Fig. 2A). In contrast, post-treatment tumor growth was not observed by the end of observation in the combined treatment group. We next investigated EGFR and CD44 expression in tumors that were removed 42 days after treatment termination by immunohistochemistry. These results demonstrated that EGFR (Fig. 2B) and CD44 (Fig. 2C) were more highly expressed in the tumor tissues from the gefitinib-only treated mice compared with the combined treatment mice. In the tumor tissues from the gefitinib-only treated mice, both EGFR and CD44 were clearly expressed along the cell membrane (Fig. 2B and C).

Combined SN-38 and gefitinib treatment inhibits CD44 expression in HNSCC cells. Next, we examined the proportion of CD44-positive cells in different HNSCC cell lines. These data showed that ~30% of HSC-2 cells were CD44-

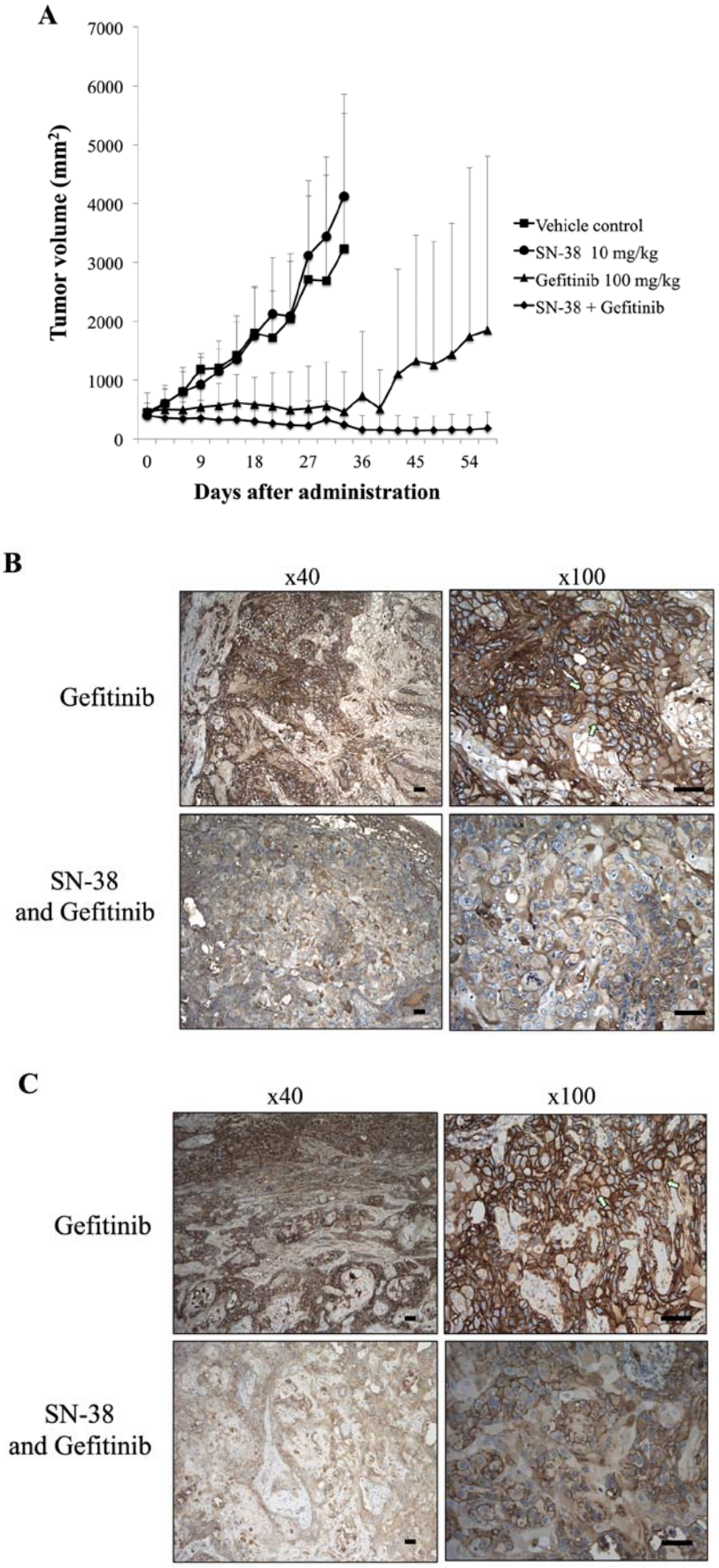

Figure 2. Effect of combined SN-38 and gefitinib administration on HNSCC using a mouse xenograft model. The intraperitoneal administration of SN-38 (10 mg/kg), and subcutaneous administration of gefitinib (100 mg/kg) and their combined therapy was carried out for 3 weeks (5 days/week), after tumors reached 400-500 mm^3. Tumor size was observed up to 60 days, even after completing the drug administration. Error bars indicate standard deviation (SD); n=5 (A). After gefitinib-only or combined therapy, tumors were examined by immunohistochemistry for EGFR (B) and CD44 (C). Scale bar indicates 100 μm. Arrows indicate representative cell staining.

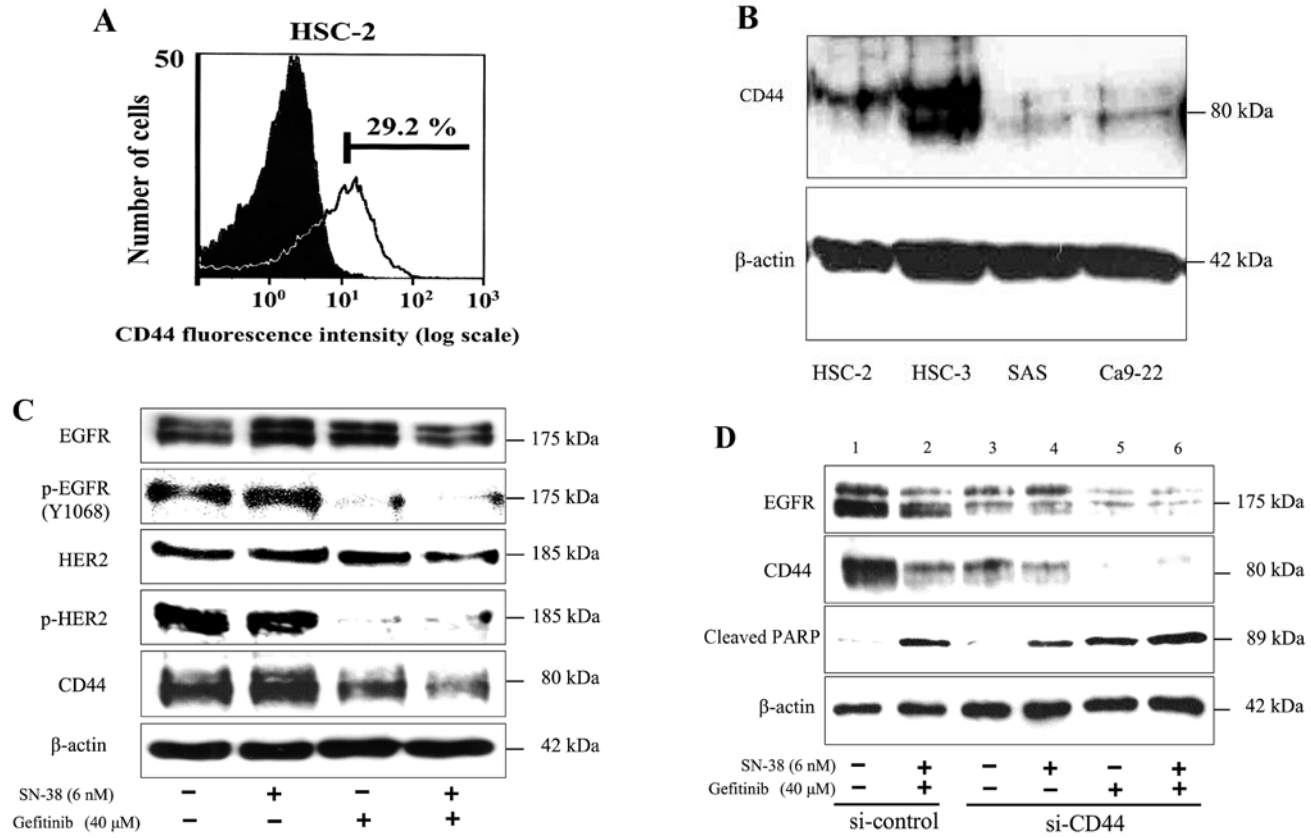

Figure 3. CD44 expression decreased following combined SN-38 and gefitinib chemotherapy *in vitro*. Representative single-parameter diagrams showing the percentage of CD44-expressing HSC-2 cells by flow cytometry (A). CD44 expression in the HNSCC cell lines HSC-2, HSC-3, SAS and Ca9-22 are shown by immunoblotting (B). EGFR, HER2, their phosphorylated forms and CD44 are shown by immunoblotting after the indicated treatments (C). EGFR, CD44 and cleaved PARP levels in siRNA-mediated CD44 knockdown cells under the indicated treatments are shown by immunoblotting (D).

Table III. Cell cycle distributions of HSC-2 cells following SN-38, gefitinib or combined treatment. Cell cycle distribution: average (%) ± SD.

Cell cycle phase	Control	SN-38 (6 nM)	Gefitinib (40 μM)	Combination
G0+G1	66.5±7.2	51.8±2.4	80.7±2.0	76.0±2.0
S	15.8±2.3	21.4±2.6	4.2±0.3	7.6±2.3
G2+M	16.9±8.1	26.7±4.5	14.8±2.0	15.1±4.1

positive (Fig. 3A). We also found that CD44 expression was higher in HSC-3 cells than in HSC-2 cells, while the SAS and Ca9-22 cell lines had less CD44 expression than HSC-2. In summary, CD44 expression was confirmed in all 4 HNSCC cell lines (Fig. 3B).

We then examined the molecular mechanism by which the combined treatment inhibited HNSCC cell proliferation. In accordance with previously published results, cell cycle analysis revealed that SN-38 decreased the G0/G1 phase from 66 to 52%, while the S phase population increased from 16 to 21% (Table III). Following gefitinib treatment, the percentage of G0/G1 phase HSC-2 cells was significantly increased to 81%, and the S phase population was significantly decreased to 4% (Table III). The combination treatment group showed the same tendency as gefitinib, and there were no significant differences between the two (Table III). EGFR and HER2 phosphorylation were inhibited by the gefitinib single treatment, and there was no significant difference between the gefitinib single treatment and the combined treatment (Fig. 3C). However, CD44 expression was inhibited in the combined gefitinib and SN-38 group (Fig. 3C, lane 4). Furthermore, when CD44 expression was inhibited by siRNA transfection (Fig. 3D compare lanes 1 and 3), EGFR expression was also inhibited (Fig. 3D, lane 3). In the context of CD44 silencing, we were able to further reduce expression of the remaining protein by SN-38 or gefitinib treatment alone (Fig. 3D, compare lanes 4 and 5 with lane 3). Notably, when the combined treatment was applied to CD44-knockdown cells, CD44 and EGFR were more strongly inhibited, and apoptosis was also induced to a greater extent (Fig. 3D, lane 6). These data revealed that combined SN-38 and gefitinib treatment inhibited CD44 expression *in vitro*.

CD44-positive HNSCC cells have characteristics of cancer stem-like cells. It has been reported that CD44 expression in HNSCC cells is a marker of cancer stem cells that has a significant relationship with prognosis (14,22). Our data showed that CD44 expression was markedly high in the growing tumor tissues from xenograft models post-treatment (Fig. 2C). Therefore, we next examined whether CD44-positive HNSCC cells showed cancer stem cell characteristics. CD44-positive cells that were isolated by the MACS

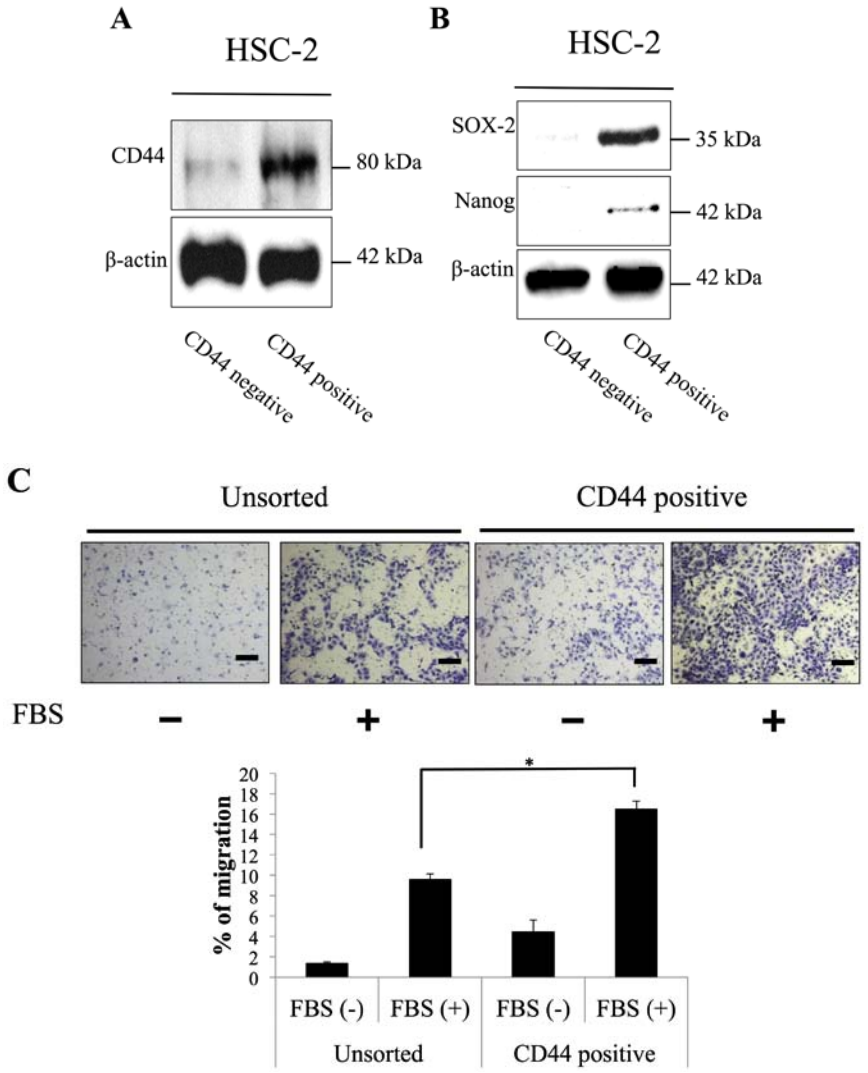

Figure 4. Properties of CD44-positive HSC-2 cells. CD44-positive and CD44-negative HSC-2 cells were separated using the MACS magnetic bead system (A). SOX-2 and Nanog expression in CD44-positive and CD44-negative HSC-2 cells was examined by immunoblotting (B). Migration assays using CD44-positive and unsorted HSC-2 cells. The percentage of migrated cells is shown in the bar graph (C). Scale bar indicates 100 μm. *P<0.01, compared with the unsorted group.

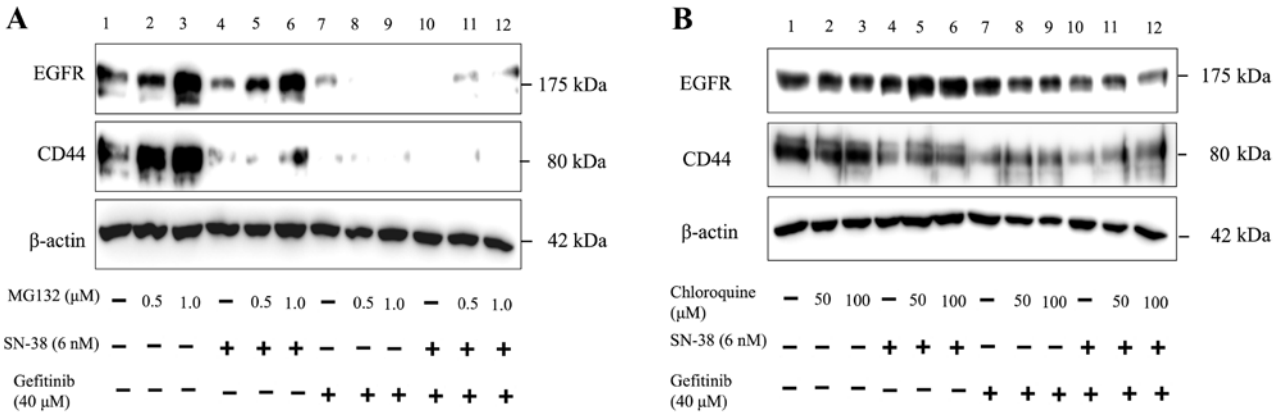

Figure 5. Examination of the reduced EGFR and CD44 expression in the combined treatment group with proteasome and lysosome inhibitors. Immunoblots showing changes in EGFR and CD44 expression by combined SN-38 and gefitinib treatment following MG132 pretreatment. The MG132 concentrations were 0.5 or 1.0 μM (A). Immunoblots showing changes in EGFR and CD44 expression by combined SN-38 and gefitinib treatment following chloroquine pretreatment. The chloroquine concentrations were 50 or 100 μM (B).

magnetic bead system strongly expressed SOX-2 and Nanog, which are known transcription factors expressed in stem cells (23,24) (Fig. 4A and B). Moreover, CD44-positive cells showed significantly increased migratory ability (Fig. 4C).

These data revealed that CD44-positive HNSCC cells have characteristics of cancer stem-like cells.

Lysosome inhibition enhances CD44 expression in HNSCC cells. Next, we examined how the combination treatment affects CD44 expression, testing the hypothesis that it may promote CD44 degradation. Therefore, we tested whether the reduction in CD44 and EGFR expression could be recovered by inhibiting the proteasome. These results showed that the reduced EGFR expression following treatment was recovered, and the reduction of CD44 by SN-38 single treatment was partially recovered when combined with MG132 (Fig. 5A, compare lanes 6 and 4). However, the reduction of CD44 expression by gefitinib single treatment (Fig. 5A, compare lanes 9 and 7) and the combination treatment were not recovered by MG132 pretreatment (Fig. 5A, compare lanes 12 and 10). Next, we investigated whether the reduction of CD44 and EGFR expression could be recovered when the lysosome was inhibited. There was no effect on EGFR expression when lysosomes were inhibited by chloroquine in the absence of the chemotherapy drugs (Fig. 5B). In contrast, the reduction of CD44 expression by gefitinib treatment alone and gefitinib plus SN-38 treatment was partially recovered by chloroquine (Fig. 5B, compare lanes 12 and 10).

Discussion

Initially, we used existing chemotherapeutic agents to identify an effective combination that inhibited HNSCC cell proliferation and induced apoptosis. Among those tested, SN-38, an active metabolite of irinotecan hydrochloride, and gefitinib, an EGFR tyrosine kinase inhibitor, were found to inhibit cell proliferation and induce apoptosis in HNSCC cells in a synergistic manner (Fig. 1). We then tested the effectiveness of this combination therapy on an *in vivo* xenograft mouse model. Contrary to our expectations, there was no significant difference in tumor growth inhibition between gefitinib treatment and combined gefitinib and SN-38 administration (Fig. 2A). However, by continuing tumor measurements after terminating chemotherapy, we found that tumors from the gefitinib-only treatment group resumed growth. Conversely, tumors from the combined treatment group did not show growth after treatment had stopped. Investigating tumor tissues from the gefitinib-only group that had begun to progress revealed that expression of the major cancer stem cell marker CD44 was increased in these tissues compared with tumor tissues from the combined treatment group (Fig. 2C). Therefore, we next focused on elucidating how combined SN-38 and gefitinib treatment affected CD44 expression in HNSCC cells. Our data showed that CD44 degradation following combined SN-38 and gefitinib treatment was partially blocked by pretreatment with the lysosome inhibitor chloroquine (Fig. 5B, compare lanes 12 and 10), while CD44 degradation was not blocked by pretreatment with the proteasome inhibitor MG132 (Fig. 5A, compare lanes 12 and 10). These studies clearly demonstrated that SN-38 treatment partially promoted the proteasomal degradation of CD44, while gefitinib and the combined treatment promoted lysosomal CD44 degradation (Fig. 5).

Many researchers have reported that CD44 is an important cancer stem cell marker in HNSCC and that CD44 expression is closely related to prognosis (22,25,26). It has also been shown that cancer stem cells have an increased capacity for self-renewal, drug resistance, metastasis and tumor recurrence (27). In the present study, we demonstrated that CD44-positive HNSCC cells have cancer stem-like characteristics (Fig. 4). We also showed that combined SN-38 and gefitinib treatment reduced CD44 expression in HNSCC cells. Furthermore, our data suggested that reduced CD44 expression was attributable to lysosomal CD44 degradation (Fig. 5).

However, when considering whether combined irinotecan hydrochloride (CPT-11), which becomes SN-38 when metabolized, and gefitinib treatment may be effective for clinically staged HNSCC, it should be noted that gefitinib has no significant clinical effect on HNSCC (28). In our data, the IC_{50} of HaCaT cells, which are normal human skin keratinocytes, was 0.63 μM, which is ~60 times more sensitive than HSC-2 cells, whose IC_{50} was 38.5 μM (Table I). These data indicate that the proliferation of normal squamous cells could be inhibited before HNSCC cells are inhibited. Therefore, it is expected that the combined treatment of irinotecan hydrochloride and gefitinib may not be realistic for HNSCC in the clinic.

Nevertheless, other studies have reported that lysosomal CD44 degradation in ovarian cancer cells inhibits metastasis (29). Therefore, inhibition of CD44 expression by enhancing lysosomal CD44 degradation may also inhibit HNSCC tumor metastasis. Additionally, CD44 was highly expressed in tumors that showed renewed growth after gefitinib treatment (Fig. 2), while the combination therapy, in which CD44 expression was inhibited, did not show tumor regrowth after finishing chemotherapy (Fig. 2). Therefore, the reduced CD44 expression due to enhanced lysosomal degradation may inhibit tumor regrowth after therapy.

Studies have shown that there are several HNSCC cancer stem cell markers in addition to CD44, including ALDH (30) and CD133 (31); double-positive cells (CD44 plus ALDH or CD133) have also been shown to be more tumorigenic and prone to metastasis compared with CD44 single-positive cells. However, we did not investigate these double-positive cells in the present study. Thus, in future studies we may consider these other cancer stem cell markers. Additionally, we showed that inhibition of CD44 expression may inhibit tumor regrowth after treatment, but it may be necessary to verify this finding in greater detail in the future.

In conclusion, we demonstrated that combined SN-38 and gefitinib treatment enhanced the lysosomal degradation of CD44, which is a known marker of cancer stem cells. This combination chemotherapy was also shown to inhibit tumor regrowth after treatment. Together, these data indicate the potential for chemotherapies that enhance the lysosomal degradation of CD44 to inhibit tumor metastasis and recurrence.

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References

1. Sanderson RJ and Ironside JA: Squamous cell carcinomas of the head and neck. BMJ 325: 822-827, 2002.
2. Stewart JS, Cohen EE, Licitra L, Van Herpen CM, Khorprasert C, Soulieres D, Vodvarka P, Rischin D, Garin AM, Hirsch FR, *et al*: Phase III study of gefitinib compared with intravenous methotrexate for recurrent squamous cell carcinoma of the head and neck [corrected]. J Clin Oncol 27: 1864-1871, 2009.
3. Vermorken JB, Remenar E, van Herpen C, Gorlia T, Mesia R, Degardin M, Stewart JS, Jelic S, Betka J, Preiss JH, *et al*; EORTC 24971/TAX 323 Study Group: Cisplatin, fluorouracil, and docetaxel in unresectable head and neck cancer. N Engl J Med 357: 1695-1704, 2007.
4. Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, *et al*: Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 359: 1116-1127, 2008.
5. Visvader JE and Lindeman GJ: Cancer stem cells in solid tumours: Accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755-768, 2008.
6. Zöller M: CD44: Can a cancer-initiating cell profit from an abundantly expressed molecule? Nat Rev Cancer 11: 254-267, 2011.
7. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA and Lander ES: Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 138: 645-659, 2009.
8. Major AG, Pitty LP and Farah CS: Cancer stem cell markers in head and neck squamous cell carcinoma. Stem Cells Int 2013: 319489, 2013.
9. Orian-Rousseau V: CD44, a therapeutic target for metastasising tumours. Eur J Cancer 46: 1271-1277, 2010.
10. Kajita M, Itoh Y, Chiba T, Mori H, Okada A, Kinoh H and Seiki M: Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J Cell Biol 153: 893-904, 2001.
11. Kim HR, Wheeler MA, Wilson CM, Iida J, Eng D, Simpson MA, McCarthy JB and Bullard KM: Hyaluronan facilitates invasion of colon carcinoma cells in vitro via interaction with CD44. Cancer Res 64: 4569-4576, 2004.
12. Golshani R, Lopez L, Estrella V, Kramer M, Iida N and Lokeshwar VB: Hyaluronic acid synthase-1 expression regulates bladder cancer growth, invasion, and angiogenesis through CD44. Cancer Res 68: 483-491, 2008.
13. Ohkoshi E and Umemura N: Induced overexpression of CD44 associated with resistance to apoptosis on DNA damage response in human head and neck squamous cell carcinoma cells. Int J Oncol 50: 387-395, 2017.
14. Joshua B, Kaplan MJ, Doweck I, Pai R, Weissman IL, Prince ME and Ailles LE: Frequency of cells expressing CD44, a head and neck cancer stem cell marker: Correlation with tumor aggressiveness. Head Neck 34: 42-49, 2012.
15. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, *et al*: The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med 17: 211-215, 2011.
16. Chou TC: Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70: 440-446, 2010.
17. Chou TC: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58: 621-681, 2006.
18. Guo B, Cao S, Tóth K, Azrak RG and Rustum YM: Overexpression of Bax enhances antitumor activity of chemotherapeutic agents in human head and neck squamous cell carcinoma. Clin Cancer Res 6: 718-724, 2000.
19. Stewart CF, Leggas M, Schuetz JD, Panetta JC, Cheshire PJ, Peterson J, Daw N, Jenkins JJ III, Gilbertson R, Germain GS, *et al*: Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice. Cancer Res 64: 7491-7499, 2004.
20. Azrak RG, Cao S, Durrani FA, Toth K, Bhattacharya A and Rustum YM: Augmented therapeutic efficacy of irinotecan is associated with enhanced drug accumulation. Cancer Lett 311: 219-229, 2011.
21. Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, Beijersbergen RL, Bardelli A and Bernards R: Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. Nature 483: 100-103, 2012.
22. Kokko LL, Hurme S, Maula SM, Alanen K, Grénman R, Kinnunen I and Ventelä S: Significance of site-specific prognosis of cancer stem cell marker CD44 in head and neck squamous-cell carcinoma. Oral Oncol 47: 510-516, 2011.
23. Qin D, Gan Y, Shao K, Wang H, Li W, Wang T, He W, Xu J, Zhang Y, Kou Z, *et al*: Mouse meningiocytes express Sox2 and yield high efficiency of chimeras after nuclear reprogramming with exogenous factors. J Biol Chem 283: 33730-33735, 2008.
24. Ang YS, Tsai SY, Lee DF, Monk J, Su J, Ratnakumar K, Ding J, Ge Y, Darr H, Chang B, *et al*: Wdr5 mediates self-renewal and reprogramming via the embryonic stem cell core transcriptional network. Cell 145: 183-197, 2011.
25. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF and Ailles LE: Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 104: 973-978, 2007.
26. Faber A, Barth C, Hörmann K, Kassner S, Schultz JD, Sommer U, Stern-Straeter J, Thorn C and Goessler UR: CD44 as a stem cell marker in head and neck squamous cell carcinoma. Oncol Rep 26: 321-326, 2011.
27. Giancotti FG: Mechanisms governing metastatic dormancy and reactivation. Cell 155: 750-764, 2013.
28. Cohen EE, Kane MA, List MA, Brockstein BE, Mehrotra B, Huo D, Mauer AM, Pierce C, Dekker A and Vokes EE: Phase II trial of gefitinib 250 mg daily in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. Clin Cancer Res 11: 8418-8424, 2005.
29. Haakenson JK, Khokhlatchev AV, Choi YJ, Linton SS, Zhang P, Zaki PM, Fu C, Cooper TK, Manni A, Zhu J, *et al*: Lysosomal degradation of CD44 mediates ceramide nanoliposome-induced anoikis and diminished extravasation in metastatic carcinoma cells. J Biol Chem 290: 8632-8643, 2015.
30. Chinn SB, Darr OA, Owen JH, Bellile E, McHugh JB, Spector ME, Papagerakis SM, Chepeha DB, Bradford CR, Carey TE, *et al*: Cancer stem cells: Mediators of tumorigenesis and metastasis in head and neck squamous cell carcinoma. Head Neck 37: 317-326, 2015.
31. Oliveira LR, Castilho-Fernandes A, Oliveira-Costa JP, Soares FA, Zucoloto S and Ribeiro-Silva A: CD44+/CD133+ immunophenotype and matrix metalloproteinase-9: Influence on prognosis in early-stage oral squamous cell carcinoma. Head Neck 36: 1718-1726, 2014.