# xCT expression modulates cisplatin resistance in Tca8113 tongue carcinoma cells

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**Abstract.** Tongue squamous cell carcinoma (TSCC), which is a subtype of head and neck cancer, is the most common type of oral cancer. Due to its high recurrence rate and chemoresistance, the average survival rate for patients with TSCC remains unsatisfactory. At present, cisplatin (CDDP) is utilized as the first-line treatment for numerous solid neoplasms, including TSCC. CDDP resistance develops in the majority of patients; however, the mechanism of such resistance remains unknown. Therefore, the present study aimed to clarify the mechanism of CDDP resistance and attempted to reduce chemoresistance. The results indicated that CDDP significantly increased expression of xCT, which is the light chain and functional subunit of the glutamate/cysteine transporter system $x_c^-$, and a subsequent increase in glutathione (GSH) levels was observed. The present study demonstrated that the upregulation of xCT expression and intercellular GSH levels contributed to CDDP resistance in TSCC cells. Furthermore, xCT suppression, induced by small interfering RNA or pharmacological inhibitors, sensitized TSCC cells to CDDP treatment. In conclusion, the present study revealed that CDDP-induced xCT expression promotes CDDP chemoresistance, and xCT inhibition sensitizes TSCC cells to CDDP treatment. These results provide a novel insight into the molecular mechanisms involved in TSCC cell chemoresistance.

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*Abbreviations:* TSCC, tongue squamous cell carcinoma; CDDP, cisplatin; Nrf2, nuclear factor erythroid 2-related factor 2; ATF4, activating transcription factor 4; SASP, sulfasalazine; GSH, glutathione; ER, endoplasmic reticulum; ARE, antioxidant response element; AARE, amino acid response element

*Key words:* tongue carcinoma cells, cisplatin, xCT, sulfasalazine, Nrf2, ATF4

## Introduction

Tongue carcinoma, which is a subtype of head and neck cancer, is the most frequently occurring oral cancer, of which , ~90% is tongue squamous cell carcinoma (TSCC) (1). Currently, TSCC is the tenth most common solid neoplasm worldwide, with a comparatively low median survival rate (5-year survival rate, ~50%) (2). Recent studies have reported that chemotherapy decreases tumor size and reduces distant metastasis in TSCC patients (3,4). Cisplatin (CDDP) is an efficacious antineoplastic compound, which is used for the treatment of a wide range of solid neoplasms, including non-small cell lung, ovarian, bladder, and head and neck carcinoma (5-8). However, the cytotoxicity caused by CDDP in TSCC chemotherapy remains unsatisfactory (9), and the exact mechanisms of CDDP-induced cytotoxicity have not been identified to date. CDDP resistance is the major obstacle that prevents successful treatment of TSCC (10). Recently, it has been reported that not only DNA-damaging stress but also endoplasmic reticulum (ER) and oxidative stress are involved in the therapeutic effect of CDDP (11,12). In particular, TSCC cells with high glutathione (GSH) levels exhibit marked resistance to CDDP treatment (13). Thus, the association between CDDP resistance and GSH-associated pathways requires investigation.

GSH is a triple peptide molecule consisting of cysteine, glutamic acid and glycine residues that is critical for the maintenance of the intracellular redox balance and detoxification (14). As a result, GSH levels exhibit a positive correlation with chemotherapy resistance, including resistance to CDDP (14,15). Numerous studies have revealed that CDDP resistance is associated with increased GSH levels and decreased reactive oxygen species (ROS) levels (15-17). However, the association between increased GSH levels and CDDP treatment, as well as CDDP-induced cytotoxicity in tongue carcinoma cells, remains unclear.

Cysteine is predominantly captured from the extracellular environment by system $x_c^-$ and it functions as the essential raw material for intercellular GSH synthesis (15). System $x_c^-$ consists of a light chain, xCT/solute carrier family 7 A11, and its cell surface subunit 4F2hc/cluster of differentiation (CD)98 (18). The specific function of system $x_c^-$ is determined by xCT (18) and thus, intracellular GSH levels are closely associated with the expression and function of xCT (19). Increasing evidence has revealed that xCT is expressed in various malignant tumors, and its expression is associated

with the development of preneoplastic lesions and cancer, poor prognosis and drug resistance (20-23). Notably, xCT is important in maintaining high levels of GSH and contributes to CDDP resistance of ovarian cancer cell lines (15). In addition, xCT is markedly upregulated in resected tongue carcinoma specimens (24). Ye *et al* (19) demonstrated that proteasome inhibitor-induced xCT expression is positively modulated by the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and activating transcription factor 4 (ATF4) via the antioxidant response element (ARE) and amino acid response element (AARE) on the promoter human *xCT* gene (19). In addition, Nrf2 and ATF4 are also induced by CDDP (25,26), while the mechanism of CDDP-inducible xCT expression and the association between that and CDDP resistance of TSCC cells remains unclear.

A series of compounds exhibit xCT inhibition, including the substrate inhibitor glutamine acid and the non-substrate inhibitor sulfasalazine (SASP) (27). SASP, a sulfa immunosuppressant that is widely used in the treatment of rheumatoid arthritis and inflammatory bowel diseases, is also an effective pharmacological inhibitor of xCT (28). Combined treatment with SASP increases the efficacy of several chemotherapeutic drugs, including gemcitabine, 5-fluouracil, bortezomib and doxorubicin, to lung adenocarcinoma cells (19,29,30). SASP has also been reported to decrease CDDP resistance in small cell lung cancer cell lines (31). However, further study regarding the combined effect of SASP and CCDP on tongue carcinoma cell lines is required.

In the present study, the mechanism of CDDP-inducible xCT expression and the function of xCT upregulation in CDDP resistance in the Tca8113 tongue carcinoma cell line were investigated. xCT expression was robustly induced by CDDP in an Nrf2 and ATF4 activation-dependent manner. CDDP-induced cells death was increased when xCT was suppressed by small interfering RNA (siRNA) or its pharmacological inhibitor. These results indicate that CDDP-prompted xCT activation increases the resistance of tongue cancer cells to CDDP treatment, and the combination of CDDP and xCT inhibitor could benefit tongue cancer chemotherapy.

## Materials and methods

*Cell line*. The human TSCC cell line Tca8113 was obtained from the China Center for Type Culture Collection (Wuhan, China). Tca8113 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Life Technologies; Thermo Fisher Scientific, Inc.) at 37˚C with in an atmosphere of 5% $CO_2$.

*Reagents*. CDDP and SASP (Sigma-Aldrich) were dissolved in 100% dimethyl sulfoxide (DMSO; cat. no. 046-21981; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and diluted to the appropriate concentrations (CDDP, 20 mg/ml and SASP, 300 mM) with culture medium prior to each experiment. The final DMSO concentration was <0.1% for the cell experiments. Rabbit polyclonal anti-xCT antibody (cat. no. ab37185) was purchased from Abcam (Cambridge, MA, USA). Anti-4F2hc/CD98 (cat. no. sc-9160) and anti-β-actin antibodies (cat. no. sc-47778) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit polyclonal anti-CD44 variant (v) antibody (cat. no. 3578) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was isolated from Tca8113 cells using the Total RNA Purification kit (Norgen Biotek Corporation, Thorold, ON, Canada), according to the manufacturer's protocol. Complementary DNA was obtained from 1 $\mu$g total RNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RT-qPCR analyses were performed using the iTaq Universal SYBR Green kit (Bio-Rad Laboratories, Inc.) and CFX Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.), under the following cycling conditions: 98˚C for 1 min, followed by 45 cycles at 98˚C for 5 sec and 60˚C for 30 sec. The designed RT-qPCR primers (Sigma-Aldrich) were as follows: Forward (F), 5'-GCTGTGATATCCCTGGCATT-3' and reverse (R), 5'-GGCGTCTTTAAAGTTCTGCG-3' for xCT; F, 5'-CCAGGTTCGGGACATAGAGA-3' and R, 5'-GAGCCTTGCCTGAGACAAAC-3' for 4F2hc/CD98; F, 5'-AGAAGGTGTGGGCAGAAGAA-3' and R, 5'-AAATGCACCATTTCCTGAGA-3' for CD44v; F, 5'-CGGTATGCAACAGGACATTG-3' and R, 5'-ACTGGTTGGGGTCTTCTGTG-3' for Nrf2; F, 5'-CTTACGTTGCCATGATCCCT-3' and R, 5'-GAGAACACCTGGAGATGGGA-3' for ATF4; and F, 5'-TGAAGGTCGGAGTCAACGATTTGGT-3' and R, 5'-GAAGATGGTGATGGGATTTC-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was used as an internal control, and the expression of the target gene was normalized relative to the expression of GAPDH. Non-specific amplification and primer dimers were examined by dissociation curves at the end of the PCRs. Data was assessed according to the comparative Cq method ($2^{-\Delta\Delta Cq}$) (19).

*Western blot analysis*. Tca8113 cells were seeded at a density of $1.5x10^5$ cells/well in 12-well plates (cat. no. CLS3513; Sigma-Aldrich), and 24 h later, cells were lysed with CelLytic M Cell Lysis Reagent (Sigma-Aldrich) for whole-cell protein extraction. Genomic DNA was sonicated for 15 sec with an output frequency of 20 kHz and 50% amplitude, using a 150VT sonicator (Biologics, Inc, Cary, NC, USA). Protein concentration was measured using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Sample buffer (4X) and 1% β-mercaptoethanol (β-ME; Wako Pure Chemical Industries, Ltd.) were added to equivalent amounts of protein (10 $\mu$g), and the samples were incubated at 95˚C for 5 min. The samples were next subjected to electrophoresis on an 8% (v/v) sodium dodecyl sulfate-polyacrylamide gel (cat. no. WT0081BOX; Thermo Fisher Scientific, Inc.), and transferred to a polyvinylidene difluoride hybridization transfer membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked at room temperature with Blocker BLOTTO Blocking Buffer (Thermo Fisher Scientific, Inc.), washed 3 times with Tris-buffered saline containing 0.05% Tween (TBST; cat. no. 206-19131; Wako Pure Chemical Industries, Ltd.), and incubated with anti-xCT, anti-4F2hc, anti-CD44v and anti-β-actin antibodies overnight at 4˚C. All the above primary antibodies were diluted in 1:1,000 with 1X TBST with 5% bovine serum albumin (cat. no. A9418; Sigma-Aldrich). Following

3 washes with TBST, the membranes were incubated for 1 h at room temperature with goat anti-rabbit immunoglobulin (Ig)G-horseradish peroxidase-(HRP) (cat. no. sc-2054; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG-HRP (cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) secondary antibodies diluted 1:10,000 with 1X TBST, and visualized in a ChemiDoc MP system (Bio-Rad Laboratories, Inc.) using the ImmunoStar LD chemiluminescence system (Wako Pure Chemical Industries, Ltd.).

*Plasmid construction and luciferase activity assay*. Human xCT gene promoter-luciferase wild-type and mutant reporter plasmids were constructed as reported previously (19). For the luciferase activity assay, Tca8113 cells were seeded in 12-well plates at a density of $0.5x10^5$ cells/well for 24 h prior to transfection. The cells were co-transfected with 0.9 $\mu$g luciferase reporter plasmids and 0.1 $\mu$g pRL-TK (internal control; cat. no. E2241; Promega Corporation, Madison, WI, USA) using Lipofectamine LTX Reagent (Life Technologies; Thermo Fisher Scientific, Inc.). The luciferase activity assay was performed 24 h subsequent to transfection using the Dual-Luciferase® Reporter Assay System (Promega Corporation).

*Intracellular GSH level determination*. GSH was derivatized using monobromobimane (mBBr; Life Technologies; Thermo Fisher Scientific, Inc.) and separated by reverse-phase high-performance liquid chromatography as previously described (32). Briefly, Tca8113 cells were collected with phosphate-buffered saline (cat. no. 1662403; Bio-Rad Laboratories, Inc.) and lysed in 0.2 M 5-sulfosalicylic acid (cat. no. 197-04582; Wako Pure Chemical Industries, Ltd.) on ice for 10 min. Samples were separated by centrifugation at 8,000 x g for 5 min. Next, mBBr was added to the supernatant fraction and allowed to react in the dark at room temperature for 30 min, and then the absorbance at 490 nm was detected with an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.). The precipitate was diluted in 0.1 N NaOH (cat. no. 1310-73-2; Wako Pure Chemical Industries, Ltd.), and protein determination was performed using the Pierce Coomassie (Bradford) Protein Assay kit (Thermo Fisher Scientific, Inc.). Bovine gamma globulin (cat. no. 5000208; Bio-Rad Laboratories, Inc.) was used for the standard curve.

*siRNA transfection of Tca8113 cells*. Human Nrf2 siRNA#1 (cat. no. 107966) and #2 (cat. no. 115762); human ATF4 siRNA #1 (cat. no. 122168) and #2 (cat. no. 122372); human xCT siRNA (cat. no. 108518); and negative control siRNA (cat. no. AM4611) were purchased from Life Technologies (Thermo Fisher Scientific, Inc.). Tca8113 cells were seeded in 12-well plates at a density of $1.5x10^5$ cells/well.. The following day, the cells were transfected with siRNA using Lipofectamine RNAiMax (Life Technologies; Thermo Fisher Scientific, Inc.). Upon 24 h incubation, the transfected cells were treated with CDDP for the indicated concentrations and times.

*Cell viability assay*. Tca8113 cells were seeded in 96-well plates ($1.5x10^4$ cells/well) prior to be subjected to transfection with xCT, Nrf2 or ATF4 siRNA, or to treatment with SASP/β-ME for 24 h, followed by incubation with 5, 10, 20, 30 or 40 $\mu$g/ml CDDP for an additional 48 h. Cell viability was measured using Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich). Briefly, the culture medium was replaced following CDDP treatment, and 10 $\mu$l CCK-8 was added. Following incubation for 45 min at 37˚C, the absorbance was measured at a wavelength of 450 nm using a microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

*Statistical analysis*. Data are presented as the mean ± standard error of the mean. Statistical analysis was conducted using one-way analysis of variance. All statistical analyses were performed using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*CDDP induces xCT expression in Tca8113 cells*. To elucidate the function of xCT in CDDP resistance, CDDP-inducible messenger RNA (mRNA) and protein xCT expression levels were investigated. As indicated in Fig. 1A-C, CDDP significantly increased xCT mRNA and protein expression levels in a time- and concentration-dependent manner, and 20 $\mu$g/ml CDDP induced maximal xCT expression following 6-h treatment. Therefore, this concentration of CDDP and treatment time was selected for subsequent experiments. CDDP induced xCT upregulation and a marginal increase in the expression of the heavy chain of system $x_c^-$ (4F2hc/CD98) 4F2hc, which may be due to a positive feedback as a consequence of xCT induction (Fig. 1A-C). Ishimoto *et al* (33) indicated that CD44v interacts with and stabilizes xCT, thus regulating the redox status and promoting tumor growth, however no significant differences in CD44v expression were observed in the present study following CDDP treatment in Tca8113 cells (Fig. 1C and D). These results demonstrated that CDDP treatment significantly increased xCT expression in Tca8113 cells.

*CDDP enhances xCT expression in an Nrf2 and ATF4-dependent manner*. Firstly, to clarify whether CDDP-induced xCT expression was dependent on Nrf2 and ATF4, the knockdown efficiency of siRNA transfection was confirmed. As shown in Fig. 2A and B, Nrf2 and ATF4 expression was effectively inhibited by individual siRNAs in the DMSO- and CDDP-treated Tca8113 cells. Two types of siRNA, which targeted Nrf2 or ATF4, were used to prevent potential off-target effects (silencing of genes other than Nrf2 and ATF4) arising from non-specific binding of the siRNAs to unrelated genes. The results demonstrated that CDDP-induced xCT expression was significantly decreased in Nrf2 and ATF4 knockdown cells (Fig. 2C and D). These results indicate that CDDP-induced xCT expression occurs in a Nrf2 and ATF4-dependent manner.

*CDDP induces xCT activation via ARE and AARE elements on the promoter of the human xCT gene*. The function of the *cis* elements on the promoter of human *xCT* in CDDP-induced xCT expression was investigated using a series of xCT gene promoter luciferase reporter plasmids. As represented in Fig. 3, CDDP induced reporter activity in the wild-type construct. Notably, constitutive and CDDP-inducible reporter

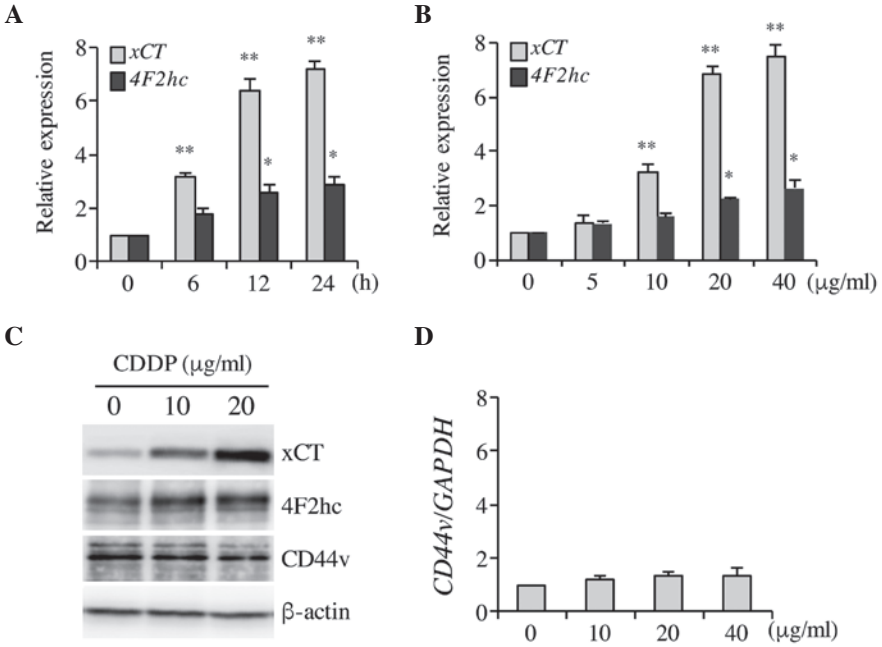


Figure 1. CDDP induced xCT expression at the mRNA and protein levels in Tca8113 cells. (A) Tca8113 cells were treated with 20 $\mu$g/ml CDDP for 24 h. Tca8113 cells were subjected to treatment with increasing concentrations of CDDP as indicated for 12 h. *xCT and 4F2hc* (B) mRNA and (C) protein expression levels were determined by RT-qPCR and immunoblotting, respectively. (D) *CD44v* mRNA expression was determined by RT-qPCR. Data are presented as the mean ± standard error of the mean of ≥3 independent experiments. $^{*}$P<0.05, $^{**}$P<0.01 vs. control. CDDP, cisplatin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CD44v, cluster of differentiation 44 variant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA.

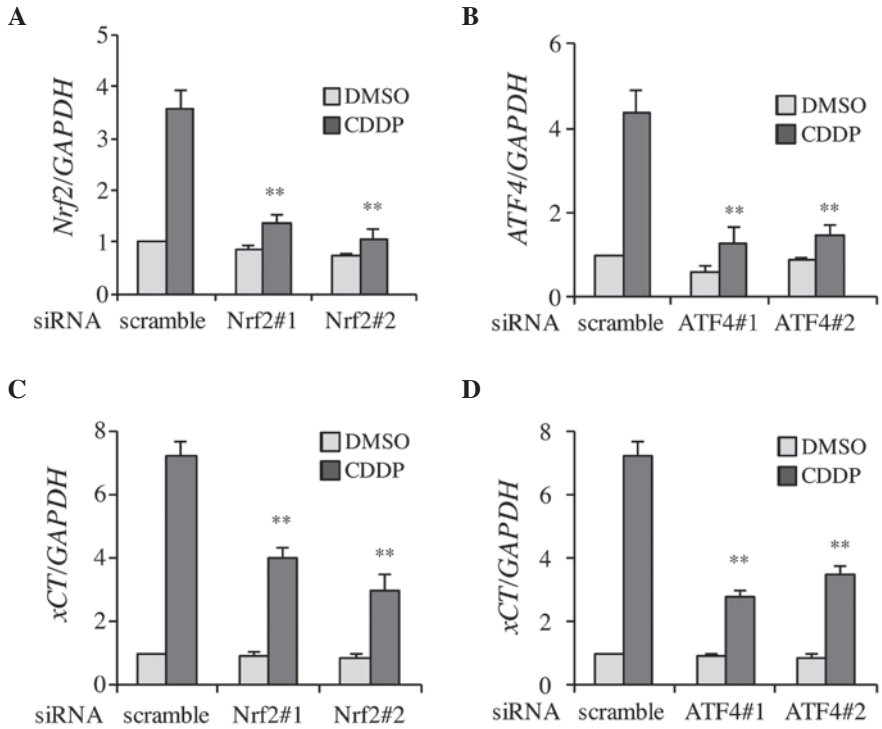


Figure 2. Nrf2 and ATF4 knockdown attenuated CDDP-induced xCT expression. (A and B) Tca8113 cells were transfected with siRNAs targeting (A) Nrf2 or (B) ATF4. Upon 24 h, cells were treated with 20 $\mu$g/ml CDDP for 12 h. The expression of xCT, Nrf2 and ATF4 messenger RNA was then measured using reverse transcription-quantitative polymerase chain reaction. (C and D) Tca8113 cells were transfected with siRNAs targeting (C) Nrf2 or (D) ATF4, and then treated with CDDP as described above. The expression levels of xCT was subsequently measured. Data are presented as the mean ± standard error of the mean of ≥3 independent experiments. $^{**}$P<0.01 vs. control. CDDP, cisplatin; Nrf2, nuclear factor erythroid 2-related-factor 2; ATF4, activating transcription factor 4; DMSO, dimethyl sulfoxide; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

activities were decreased in ARE, AARE-F and AARE-R mutant reporter genes, and this effect was decreased further in the AARE-F&R mutant reporter construct. CDDP-inducible reporter activity was almost eliminated in the triple mutant reporter gene. These results indicate that ARE and AARE are required for CDDP-triggered xCT induction.

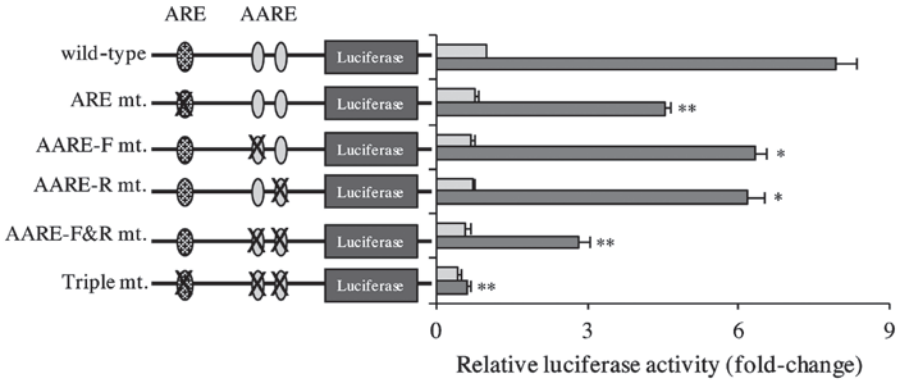


Figure 3. CDDP-inducible xCT activation was dependent on the ARE and AARE on the promoter of the human *xCT* gene. Tca8113 cells were transfected with wild-type or mutant xCT promoter luciferase reporter plasmids, and 24 h later, the culture medium was replaced with 20 $\mu$g/ml CDDP. Following 24 h, the reporter activity was determined. Data are presented as the mean ±standard error of the mean. $^{*}$P<0.05, $^{**}$P<0.01 vs. control. ARE, antioxidant response element; AARE, amino acid response element; CDDP, cisplatin; F, forward; R, reverse; mt., mutant.

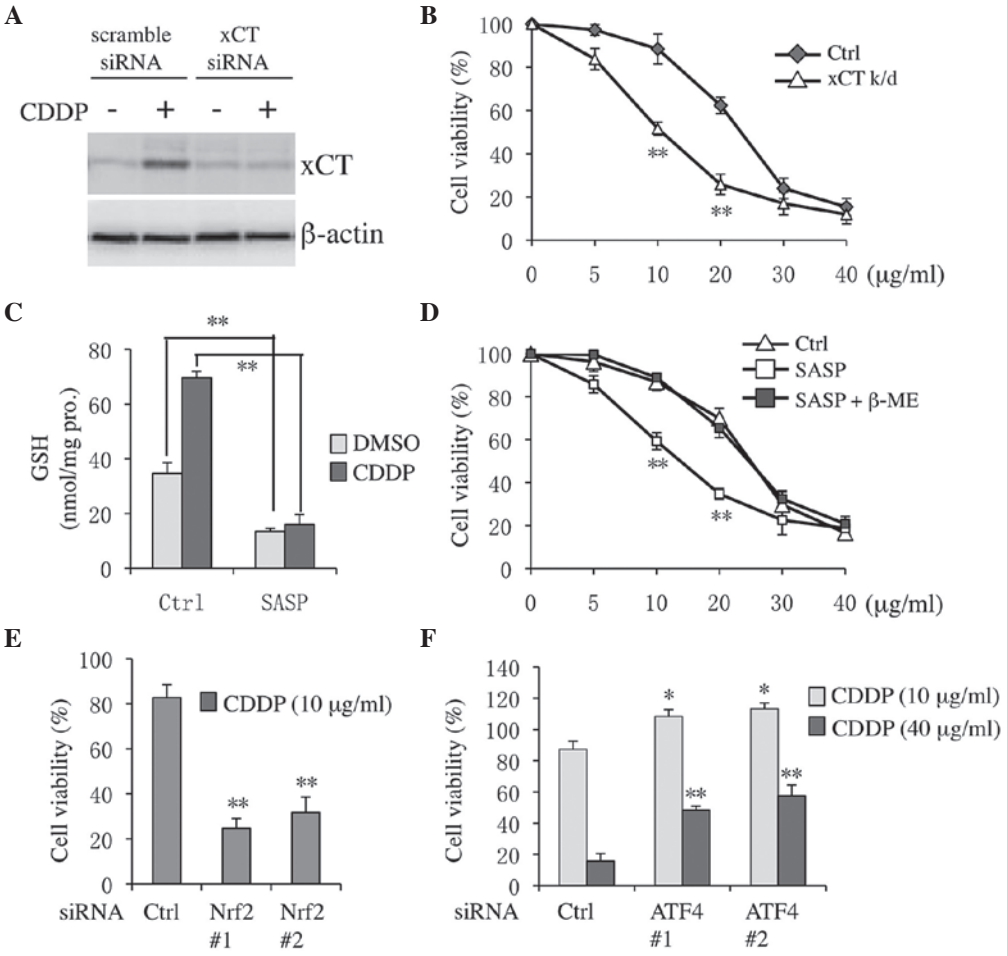


Figure 4. xCT inhibition sensitized Tca8113 cells to CDDP. (A) Tca8113 cells were treated with 20 $\mu$g/ml CDDP for 12 h following transfection with xCT or scramble siRNA. (B) Tca8113 cells were transfected with xCT or scramble siRNA for 24 h, and cells were next incubated with 0, 5, 10, 20, 30 or 40 $\mu$g/ml CDDP for an additional 48 h. Cell viability was measured with the Cell Counting Kit-8 assay. (C) Tca8113 cells were pre-treated with 0.3 mM SASP for 30 min, and then cultured with 20 $\mu$g/ml CDDP for 24 h. Subsequently, intercellular glutathione levels were estimated. (D) Tca8113 cells were treated with SASP and 0-40 μg/ml CDDP for 48 h, and cell viability was analyzed. Cells were transfected with (E) Nrf2 or (F) ATF4 siRNAs for 24 h, and then subjected to 10 or 40 $\mu$g/ml CDDP treatment for an additional 48 h. Data are presented as the mean ±standard error of the mean. $^{*}$P<0.05, $^{**}$P<0.01 vs. control. CDDP, cisplatin; siRNA, small interfering RNA; SASP, sulfasalazine; Nrf2, nuclear factor erythroid 2-related-factor 2; ATF4, activating transcription factor 4; β-ME, β-mercaptoethanol; Ctrl, control; GSH, glutathione; DMSO, dimethyl sulfoxide; k/d, knockdown.

*xCT knockdown or inhibition increases Tca8113 cell sensitivity to CDDP.* Firstly, the siRNA knockdown efficiency for targeting human xCT was confirmed. xCT expression levels were effectively decreased in both DMSO- and CDDP-treated cells (Fig. 4A). Notably, CDDP cytotoxicity was markedly increased in xCT knockdown cells compared with scramble

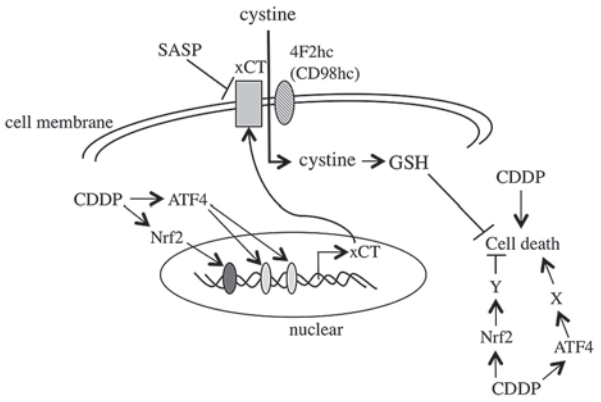


Figure 5. Hypothetical model of CDDP-induced xCT activation pathway and the mechanism of tongue squamous cell carcinoma CDDP resistance. CDDP, cisplatin; Nrf2, nuclear factor erythroid 2-related factor 2; ATF4, activating transcription factor 4; GSH, glutathione; SASP, sulfasalazine; CD98, cluster of differentiation 98.

siRNA-transfected cells (Fig. 4B). Similarly, following co-treatment with DMSO or CDDP and the xCT pharmacological inhibitor SASP, the levels of GSH were downregulated in both DMSO- and CDDP-treated cells (Fig. 4C). Furthermore, co-treatment with SASP and CDDP markedly sensitized Tca8113 cells to CDDP (Fig. 4D). The inhibitory effect of SASP was suppressed following the addition of β-ME, which could bypass xCT to allow cysteine uptake via natural amino acid transporters (19). Previously, it has been demonstrated that Nrf2 and ATF4 regulate xCT following CDDP treatment (15,18). Thus, the effect of Nrf2 and ATF4 knockdown on the sensitivity of Tca8113 cells to CDDP was also assessed in the present study. As shown in Fig. 4E, CDDP inducible cytotoxicity was markedly increased in Nrf2 knockdown cells compared with the control. By contrast, following ATF4 knockdown, cell viability increased in both high and low CDDP concentration-treatment groups compared with the control (Fig. 4F).

## Discussion

Chemotherapy is a widely used cancer treatment, in addition to surgery and radiotherapy (3). CDDP is the standard chemotherapeutic drug administered to patients with tongue carcinoma (3). However, drug resistance is common and leads to the failure of CDDP therapy (10). Thus, the mechanisms underlying tongue carcinoma cell resistance to CDDP require investigation. An increasing number of studies have indicated that CDDP resistance is associated with various factors, including various microRNAs, which modulate CDDP chemosensitivity by targeting certain genes (34,35); cell protective autophagy, which diminishes CDDP-induced apoptotic cell death (36); upregulated GSH levels; and downregulated ROS levels (15,37). In the present study, the mechanism of CDDP-triggered xCT induction and the function of xCT in tongue carcinoma cell CDDP resistance was investigated, and the conclusion is summarized in Fig. 5.

xCT is essential for GSH synthesis and maintenance, which is a critical regulator of the cellular redox state (38). Thus, xCT has been considered as a potential target for cancer treatment, including tongue carcinoma (24). Previous studies have demonstrated that SASP enhances CDDP-inducible cytotoxicity by preventing electrophiles from conjugating with GSH, indicating that combined treatment with CDDP and SASP may be beneficial (31). SASP is a well-established pharmacological inhibitor of xCT, however, the effect of SASP treatment on xCT and GSH levels has not yet been investigated (31,39). In the present study, xCT suppression increased CDDP cytotoxicity. In addition, CDDP treatment markedly increased GSH levels in Tca8113 tongue cancer cells, which was attenuated by SASP-induced xCT suppression (Fig. 4C). Furthermore, the sensitivity of Tca8113 cells to CDDP treatment increased under conditions of low GSH levels as a result of xCT inhibition caused by SASP or siRNA (Fig. 4B and D). However, the present study did not provide immediate evidence that decreased GSH levels trigger ROS accumulation and subsequent activation of the cell death signaling pathway in CDDP and SASP co-treated Tca8113 cells, although previous studies have suggested such association in various experimental conditions (40-42).

It has been reported that xCT is modulated in an Nrf2 and ATF4-dependent manner following proteasome inhibitor treatment (19). In the present study, Nrf2 and ATF4 knockdown by siRNAs attenuated CDDP-triggered xCT induction (Fig. 2). Furthermore, the binding sites ARE and AARE of Nrf2 and ATF4 are critical for CDDP-inducible xCT activation (Fig. 3). Additionally, no significant differences in the mRNA or protein expression levels of CD44v were identified following CDDP treatment in Tca8113 cells, although previous studies have indicated that CD44v may promote tumor growth by stabilizing xCT (33) and increasing CDDP resistance in head and neck squamous cells (43). It is hypothesized that CD44v may stabilize xCT; however, the association between CDDP treatment and CD44v induction remains unclear. The present authors hypothesize that CDDP-inducible xCT activation predominantly occurs via binding of Nrf2 and ATF4 to the ARE and AARE elements on the promoter of the xCT gene, rather than as a result of increased stabilization of the xCT-CD44v complex. Although the present study demonstrated that xCT is modulated by Nrf2 and ATF4 and contributes to the resistance of CDDP treatment in Tca8113 cells, the effect of Nrf2 and ATF4 suppression by siRNA was not consistent with the effects of xCT knockdown (Fig. 4E and F). Previously, Tanabe *et al* (26) reported that CDDP-inducible upregulation of ATF4 increased CDDP resistance in human KB epidermoid and prostate cancer cell lines. However, in the present study, Tca8113 cells treated with 10 or 40 $\mu$g/ml CDDP exhibited lower sensitivity to CDDP compared with the control following ATF4 knockdown (Fig. 4F). It is possible to hypothesize that an alternative gene, in addition to xCT, is downregulated in the downstream ATF4 signaling cascade, such as the C/EBP homologous protein (CHOP) (44). CHOP induction is the key factor in ER stress-induced apoptosis (45,46), and is also a repressor of Wnt/T-cell factor signaling (47), which is associated with angiogenesis, migration and survival of cancer cells (48-50). However, in the present study, the suppression of Nrf2 by siRNA exhibited the opposite effect to ATF4 suppression. It was demonstrated that Nrf2 knockdown sensitized Tca8113 cells to CDDP and exceeded the effects of xCT knockdown

(Fig. 4E). Nrf2 exhibits a critical function in the induction of phase II detoxifying enzymes, including glutamate-L-cysteine ligase catalytic subunit, nicotinamide adenine dinucleotide (P) H: quinone oxidoreductase and heme oxygenase-1, via the Kelch-like ECH-associated protein 1-Nrf2-ARE pathway, which has been reported to induce CDDP resistance in tumor cells (51,52). Although Nrf2 and its target gene contribute to CDDP resistance, the present authors postulate that it is not advisable to increase CDDP cytotoxicity via Nrf2 suppression. In various animal models (53-55), Nrf2-ARE activation has been demonstrated to prevent CDDP-induced nephrotoxicity, which is the most severe adverse effect that limits high-dose CDDP therapy (56).

In conclusion, the present study demonstrated that Nrf2/ATF4-dependent xCT induction is involved in CDDP resistance of Tca8113 tongue carcinoma cells. These results suggest that it may be beneficial to combine CDDP and pharmacological xCT inhibitors for the treatment of tongue carcinoma.

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