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Optimal Cetuximab Contact Concentration Using a Collagen Gel Droplet-Embedded Culture Drug Chemosensitivity Test in Human Oral Squamous Carcinoma Cell Lines

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Abstract

Objective: There is no established therapy for recurrent/metastatic head and neck squamous cell carcinoma (HNSCC), making decisions regarding anticancer agent use and other treatments difficult. Cetuximab, an anticancer drug used as molecular targeted therapy, is administered irrespective of patient sensitivity due to unavailability of biomarkers predicting its efficacy. We investigated the utility of antitumor effect prediction of cetuximab using collagen gel droplet-embedded culture drug sensitivity test (CD-DST).

Methods: We evaluated 13 human oral squamous carcinoma cell lines (Ca9-22, SAS, SAT, HSC-2, HSC-3, HSC-4, OSC-19, OSC-20, HO-1-N-1, HO-1-u-1, KON, SCC-4, and Nialym). The expression of cetuximab-related genes was confirmed in each cancer cell line using RT-PCR, and CD-DST was used to measure cell line sensitivity to cetuximab and to calculate an optimal contact concentration based on the HNSCC clinical response rate and efficacy rate. Furthermore, CD-DST was performed at this calculated optimal contact concentration using the treatment regimens cetuximab+cisplatin and cetuximab+cisplatin+5-fluorouracil. *In vivo* assessment was performed in nude mice that were administered cetuximab (250 μg/ml) and cisplatin alone and in combination, and the results were compared with the CD-DST results. Results: Results of RT-PCR showed no specificity. With CD-DST, the optimal contact concentration of cetuximab was 250 μg/ml. An enhanced antitumor effect was observed in some of the cell lines with low susceptibility to CDDP and CF. Antitumor effects for CD-DST and nude mouse experiments were almost equal.

Conclusions: CD-DST can indicate the clinical efficacy of cetuximab. Regarding anticancer drug groups, low-sensitivity cell lines exhibited high sensitivity for regimen including cetuximab, suggesting cetuximab may enhance the antitumor effects of existing cytotoxic anticancer drugs.

Keywords: Chemosensitivity test; Collagen gel; Droplet embedded; Culture drug sensitivity test (CD-DST); Optimal contact concentration; Molecular target drug; Cetuximab; Oral squamous cell carcinoma (OSCC)

Introduction

Recent advances in reconstructive techniques and chemotherapy following extensive surgery have improved outcomes following the primary treatment of oral squamous cell cancer (OSCC). However, there are no established methodologies for treating progressive, recurrent, or metastatic OSCCs. However, cetuximab, which binds specifically to the epidermal growth factor receptor (EGFR), is a promising novel chemotherapy drug for treating OSCC. Previous studies have demonstrated that cetuximab combined with radiotherapy improves the local disease control rate and patient survival rate for locally advanced squamous cell cancer of the head and neck (LA-SCCHN). Moreover, cetuximab treatment in combination with platinum drugs improves the survival rate in patients with recurrent or metastatic SCCHN (R/M-SCCHN) [1-4]. Cetuximab has previously been used to treat patients with curatively unresectable advanced or recurrent colorectal cancer harboring EGFR-positive and KRAS wild-type mutations, with evaluation of EGFR expression and KRAS mutation providing clinically significant predictions of therapeutic response. Therefore, colorectal chemotherapy has significantly advanced with regard to personalized medicine [5-7]. However, there are no established predictors of OSCC therapeutic response, and this inability to predict tumor response has resulted in drugs being administered to patients irrespective of tumor sensitivity. Cetuximab reportedly causes infusion reactions and other serious adverse events; therefore, identifying a predictor of OSCC patient therapeutic response is essential to eliminate ineffective drug administration and associated patient risks.

Methods for predicting the effects of anticancer drugs include analysis of multidrug resistant genes, the gene expression patterns of the enzymes associated with anticancer drug metabolism, and various anticancer drug sensitivity tests. However, none of these methods have achieved clinical application with OSCC. Chemosensitivity testing is used in the fields of colorectal, gastric, and breast cancer and is purportedly effective in personalized medicine [8]. Although chemosensitivity tests are broadly classified into categories of *in vivo* and *in vitro* methods, *in vitro* methods are mainly used because they are simple, low cost, and rapid. To date, various *in vitro* methods have been utilized, such as the human tumor clonogenic assay (HTCA) described by Salmon and Hamburger in 1979 [9], succinate dehydrogenase inhibition (SDI) [10], and thymidine incorporation assay (TIA) [11]. However, all of these methods are problematic in terms of the difficulty involved in preparing primary cell cultures, contamination by fibroblast

cells, and determining an appropriate drug contact concentration. The CD-DST method developed by Kobayashi in 1995 is a chemosensitivity test that combines a micro-three-dimensional (3D) cell culture, serum-free medium, and image-based colorimetry. This method has achieved high success rates in a range of fields by overcoming many of the issues faced by conventional chemosensitivity testing [12,13]. CCD-DST utilizes a micro-3D culture; therefore, it can be applied to evaluating OSCCs and other cancers with low tumor volume. Furthermore, its use has been described in head and neck cancer-personalized medicine initiatives as a chemosensitivity test for evaluating cytotoxic anticancer drug susceptibility [14]. However, there have been no documented attempts to use CD-DST to predict the antitumor effects of cetuximab, in part due to the test's lack of an established optimal contact concentration for OSCCs. With this in mind, we used 13 human OSCC cell lines to establish a baseline optimal contact concentration and to predict the antitumor effects of cetuximab with the aim of establishing a means to predict the drug's antitumor effects on OSCCs using CD-DST.

Materials and Methods

Test materials

The ethics committee of Nippon Dental University, School of Life Dentistry at Niigata (Approval No. ECNG-H-119) approved study. This study evaluated 13 human OSCC cell lines: Ca9-22 gingival SCC, SAS lingual SCC, SAT oral SCC, HSC-2 oral SCC, HSC-3 lingual SCC, HSC-4 lingual SCC, OSC-19 lingual SCC, OSC-20 lingual SCC, HO-1-N-1 buccal SCC, HO-1-u-1 oral floor SCC, KON oral floor SCC, SCC-4 lingual SCC, and Nialym lingual SCC. The Nialym cell line is a stably growing, sub-cultured human OSCC cell line derived from a patient that underwent surgery in our department [15]. The Ca9-22 and SAS cell lines were purchased from the Health Science Research Resources Bank, and the remaining 10 cell lines were purchased from the Japanese Collection of Research Bioresources (JCRB), Cell Bank at the National Institute of Biomedical Innovation. Cell lines were sub-cultured until use in 35 mm plastic Petri dishes containing a culture fluid composed of Dulbecco's modified eagle's medium /F12 (DMEM/F12; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% fetal bovine serum (FBS; Life Technologies, Van Allen Way, CA, USA), nonessential amino acid solution (Life Technologies), 1% penicillin-streptomycin (Life Technologies), and 0.1% fungizone (Life Technologies) placed in a 5% CO_2 incubator at 37°C and 95% humidity.

Cetuximab (Erbitux® Injection 100 mg/20 ml; Merck Serono, Tokyo, Japan), cisplatin (CDDP; Randa Injection 50 mg/100 ml; Nippon Kayaku, Tokyo, Japan), and 5-fluorouracil (5-FU injection 250 Kyowa®; Kyowa Hakko, Tokyo, Japan) were tested as representative anticancer drugs. The mice used in this study were 6-week-old female BALB/c/nu/nu strain mice (Clea Japan, Tokyo, Japan) bred under specific pathogen-free conditions.

Confirmation of cetuximab-related gene expression using reverse transcription polymerase chain reaction (RT-PCR)
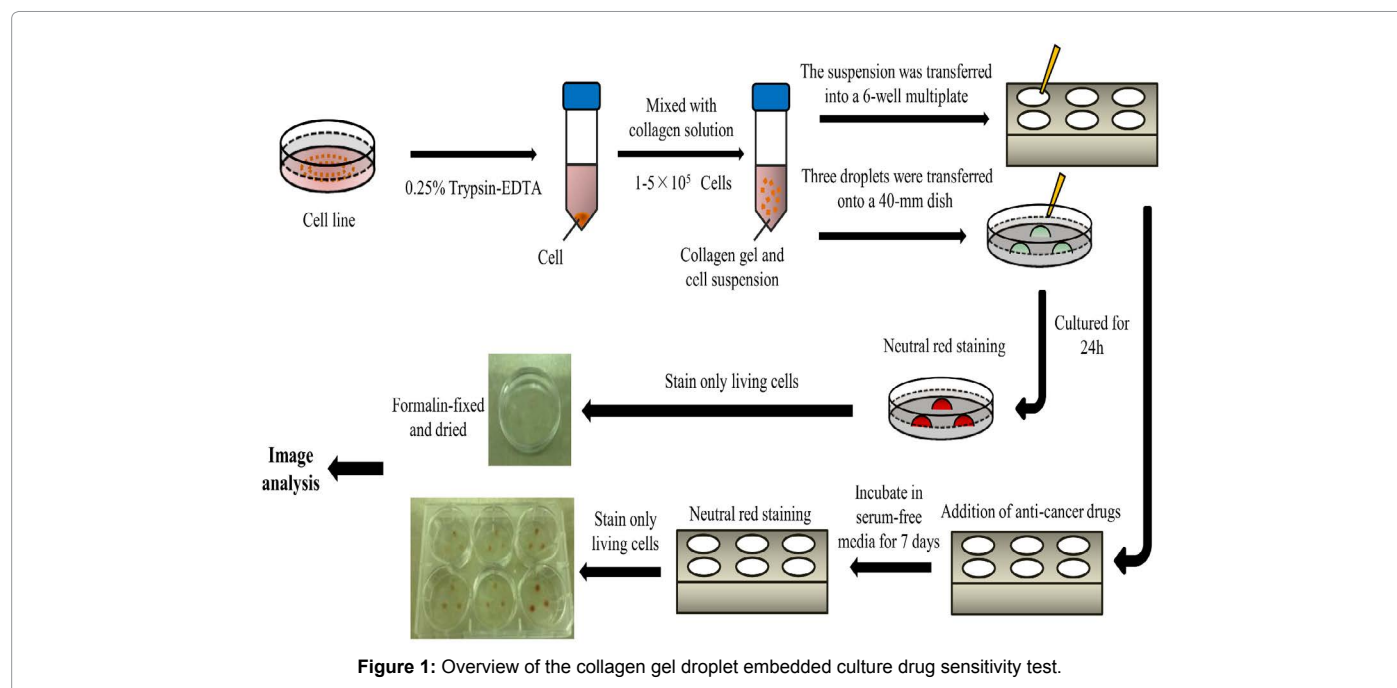
Confirmation of cetuximab-related gene expression in the 13 cell lines was performed using RT-PCR. Total RNA was isolated from each cell lines using the ISOGEN II reagent (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's extraction protocol. Using 1 µg of the extracted total RNA, cDNAs were synthesized using the high-capacity cDNA reverse transcription kit (Life Technologies). PCR amplification was performed for 35 cycles using the Platinum PCR Super Mix (Life Technologies), DNA primers for the cetuximab-related genes *EGFR*, human epidermal growth factor receptor 2 *(HER2)*, human epidermal growth factor receptor 3 *(HER3)*, and *EGFR* variant *III (EGFRvIII)*, and for the tumor suppressor gene phosphatase and tensin homolog deleted on chromosome 10 *(PTEN)* in a PCR cycle consisting of a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 60 s (Table 1). PCR amplification was also performed under the same conditions using primers for glyceraldehyde-3-phosphate dehydrogenase as an internal standard. Amplified PCR products were subjected to electrophoresis on a 2% agarose gel (Nippon Gene) and then visualized with ethidium bromide to determine the size of the amplicons.

Evaluation of cetuximab sensitivity using CD-DST

CD-DST was used for chemosensitivity testing, and the Primaster® human cancer cell primary culture kit (Kurabo Industries Ltd., Osaka, Japan) was used according to the method proposed by Kobayashi et al. [12]. Each cell line was evaluated in triplicate according to common laboratory methods (Figure 1). The test involved mixing the A solution (Cellmatrix® Type CD), B solution (10 × concentration F-12 medium), and C solution (reconstituted buffer solution) in the culture kit at a ratio of 8:1:1 and then mixing the cancer cells after adjustment to a density of $1-5 \times 10^5$ cells/ml. Using a micropipette, three drops totaling 90 µl were added to each well of a six-well multiplate (FALCON®, NY, USA). The dispensed solution was then incubated for 1 h at 37°C until it formed a gel, after which it was cultured for 24 h in DF medium containing 10% FBS. Drug contact concentration and contact times were set using conditions that resembled those used in a clinical dosing. The study evaluated six concentrations of cetuximab (10, 50, 250, 500, 750, and 1,000 µg/ml), with the upper limit of 1,000 µg/ml representing approximately 200% of the mean maximum plasma concentration under clinical dosing and a lower limit of 10 µg/ml, representing approximately 20% of the mean plasma concentration. Cells were contacted to these concentrations in individual wells for 144 h. Single wells were also contacted to CDDP and 5-FU concentrations of 0.5 µg/ml and 0.7 µg/ml for 24 h according to the method described by Sakuma et al. [16]. After contact, wells were washed with phosphate-buffered saline (PBS; TAKARA BIO INC., Shiga, Japan) to remove all residual anticancer drugs, and then the cells were cultured in serum-

	Forward Primer	Reverse Primer	Product size (bp)
EGFR	5'-CTATGAGATGGAGGAAGACG-3' 5'-TGTTTGGGACCTCCGGTCAG-3'	5'-CAGAGGAGGAGTATGTGTGA-3' 5'-GGCAGGTCTTGACGCAGTGG-3'	217 348
HER2	5'-CATTGGGACCGGAGAAACCA-3' 5'-GATTCCCCTCCATTGGGACC-3'	5'-AGGGGTGGTATTGTTCAGCG-3' 5'-TGAGTTCCAGGTTTCCCTGC-3'	553 366
HER3	5'-CATCGTGAGGGACCGAGATG-3' 5'-CACCGAGATTCTGTCAGGGG-3'	5'-TGTTGCTCGAGTCCACAGTC-3' 5'-CCCACAAGGCTCACACATCT-3'	548 556
PTEN	5'-AGCTGGAAAGGGACGAACTG-3'	5'-AGGTTTCCTCTGGTCCTGGT-3'	485
EGFRvIII	5'-AACCCCGAGGGCAAATACAG-3'	5'-AGGGAACAGGAAATATGTCGAA-3'	452
GAPDH	5'-GTCAAGGCTGAGAACGGGAA-3'	5'-GCTTCACCACCTTTCTTGATG-3'	613

Table 1: Sequence of PCR primers.

Figure 1: Overview of the collagen gel droplet embedded culture drug sensitivity test.

free medium containing EGF. After 6 days of culture in the serum-free medium, the cells in each well were stained for 2 h with a neutral red solution and then fixed for 40 min in a 10% neutral formalin solution (FUJIMI Factory Ltd., Osaka, Japan). Next, the plates were washed in water and then dried. The resulting fixed cells were evaluated for drug response using image analysis.

Evaluation of treatment response using image analysis

Drug antitumor effects were evaluated based on the method proposed by Koezuka et al. [17] using the Primage® image analysis system (Kurabo Industries Ltd., Osaka, Japan). Grayscale imaging was performed on the prepared cell materials, and differences in staining were used to screen for and eliminate from the analysis any images that did not contain cancer cells. Cancer cell proliferation and drug antitumor activity were evaluated by measuring the volume of the colonies present on the rendered cancer cell images. The ratio of anticancer drug exposure (treatment) group volume (V_C) to control group volume (V_C), or V_T/V_C, was used to determine the T/C value. A value <50% was scored as high sensitivity, whereas a value >50% was scored as low sensitivity. The criterion for evaluability was deemed to be a tumor cell growth rate that was ≥0.8 times that of the baseline growth.

Setting the optimal concentration for drug contact

The optimal contact concentration for cetuximab was set based on the method proposed by Nagai et al. [18], wherein the CD-DST-determined T/C values for the 13 cell lines were used to develop a logarithmic approximation curve based on the efficacy rate curve at each concentration, with the cumulative efficacy rate for each concentration on the y-axis and anticancer drug concentration (μg/ml) on the x-axis. Using this method, cetuximab's clinical response rate for head and neck cancers of 13% [19] was mapped onto the y-axis to calculate the optimal contact concentration.

Evaluation of sensitivity to cetuximab combination chemotherapy using CD-DST

To investigate cetuximab's ability to enhance the effects of

conventional cytotoxic anticancer drugs, CD-DST was used to evaluate cancer cell chemosensitivity to CDDP alone, CDDP+cetuximab, CDDP+5 FU, and CDDP+5 FU+cetuximab.

Antitumor effect of cetuximab in nude mice

Nude mice were used to confirm the correlation between CD-DST and clinical results. Cancer cells were subcutaneously grafted to the dorsal region of nude mice, and testing was performed on the five cell lines (Ca9-22, SAS, SAT, HSC-3, and OSC-19) that survived grafting and formed tumors. These cell lines showed successful engraftment and stable growth when subcutaneously transplanted to the dorsal region of nude mice. Tumor size ($1/2 \times$ length $\times$ breadth^2) was measured, and anticancer drug dosing was initiated when the tumors were 100-300 mm^3. Drugs were dosed using a single intraperitoneal injection of 8 mg/kg CDDP, as proposed by Kondo et al. [20], and an intraperitoneal injection of 0.25 mg cetuximab every 3 days, as proposed by Luo et al. [21], in an attempt to recreate the antitumor effects produced by clinical administration. The cancer drug antitumor effects were determined using the method suggested by Geran et al. [22] whereby T/C values were determined based on the relative tumor weights of the control group and the treatment group at 21 days after dosing. High sensitivity was defined as <50% T/C, whereas low sensitivity was defined as >50% T/C. These *in vivo* results were compared with those obtained using CD-DST. Statistical comparisons of the cetuximab and CDDP groups were performed using two-way analysis of variance (Bonferroni post-test). All animal experiments were approved by the Animal Experiment Ethical Review Board of the Nippon Dental University School of Life Dentistry at Niigata (approval no. 131).

Results

Expression of cetuximab-related genes in 13 human OSCC cell lines

EGFR, HER3, EGFRvIII, and *PTEN* were expressed by all cell lines tested, although *HER2* was not expressed by any of the cell lines. None of the 13 cell lines exhibited specific expression of cetuximab-related genes (Figure 2).

Calculation of cetuximab's optimal contact concentration using CD-DST

The CD-DST results for cetuximab, CDDP+cetuximab, and CDDP+5 FU+cetuximab are shown in Table 2. All cell lines showed low sensitivity to cetuximab at doses of 10 and 50 µg/ml. High sensitivity was observed in one cell line at 250 µg/ml, 2 cell lines at 500 µg/ml, and three cell lines at 750 and 1,000 µg/ml. The cumulative efficacy rate (%) at each concentration and the cumulative *in vitro* efficacy rate curve are presented in Figure 3. The optimal contact concentration was estimated

to be 250 µg/ml based on the logarithmic approximation curve and the clinical response rate for cetuximab monotherapy of head and neck cancers.

CD-DST evaluation of sensitivity to cetuximab combination chemotherapy

T/C values in the cetuximab+CDDP combination therapy group were lower than in the cetuximab and CDDP monotherapy groups. Moreover, *T/C* values in the cetuximab+CDDP+5-FU combination
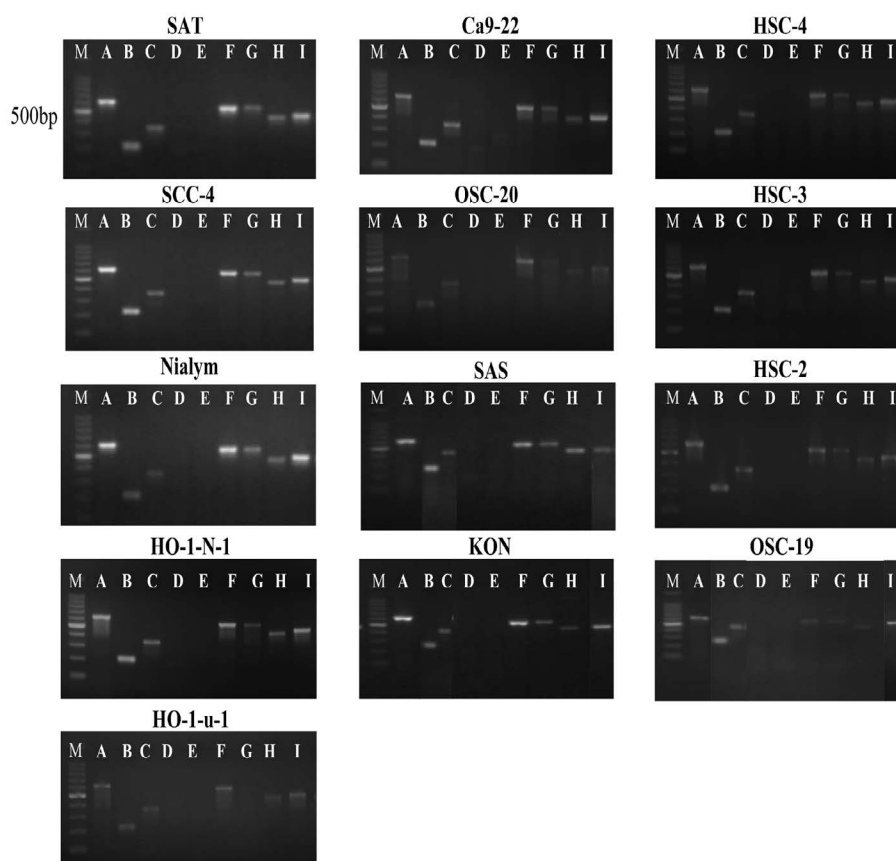
Figure 2: Results of RT-PCR amplification EGFR, HER3, EGFRvIII and PTEN were all expressed in the cell lines; however, HER2 was not expressed in any of the cell lines; A: GAPDH, B.C: EGFR, D. E: HER 2, F. G: HER 3, H: EGFRv Ⅲ;I: PTEN.

µg/mL \ Cell	Ca9-22	SAS	SAT	HSC-2	HSC-3	HSC-4	OSC-19	OSC-20	HO-1-N-1	HO-1-u-1	KON	SCC-4	Nialym	Efficacy rate (%)
10	98.5	97.8	91.8	78.9	97.4	70.0	85.0	97.0	70.9	96.7	104.1	77.3	143.9	0
50	99.4	102.6	88.6	67.6	82.4	66.3	73.3	92.9	62.1	88.4	64.0	78.9	123.1	0
250	80.4	99.9	80.4	**45.6**	75.6	53.8	68.3	92.7	51.6	97.2	63.2	75.1	153.5	7.6
500	84.2	95.8	70.0	**45.9**	74.6	56.1	64.7	77.4	**48.0**	89.1	53.5	64.0	144.9	15.3
750	78.5	62.1	64.1	**45.3**	74.6	**42.5**	63.5	81.8	**45.8**	90.7	59.3	61.7	88.3	23.0
1000	72.1	72.8	57.1	**35.0**	67.1	**37.8**	52.3	66.6	**40.3**	78.6	50.7	51.3	99.5	23.0
CDDP0.5	65.9	62.8	60.3	72.4	61.0	72.3	63.8	57.1	97.5	69.1	**43.6**	56.3	73.4	7.6
CDDP0.5+Cetuximab250	**29.1**	**26.6**	**33.9**	56.3	54.9	53.1	**43.7**	**40.0**	**23.7**	104.6	**47.4**	64.6	106.9	46.2
CDDP+5-FU	59.6	**35.6**	53.6	57.0	82.2	75.0	58.5	50.7	93.0	**45.5**	**23.3**	76.7	**47.7**	23.0
CDDP+5-FU+Cetuximab250	**38.8**	**28.7**	**32.3**	**42.0**	73.5	**30.7**	**30.0**	**46.1**	**18.6**	80.4	**17.3**	**33.3**	61.6	76.9

Table 2: Results of the CD-DST method (T/C values) T/C value <50% was scored as high sensitivity, whereas T/C value >50% was scored as low sensitivity. Boldface and underlined parts showed a high sensitivity.
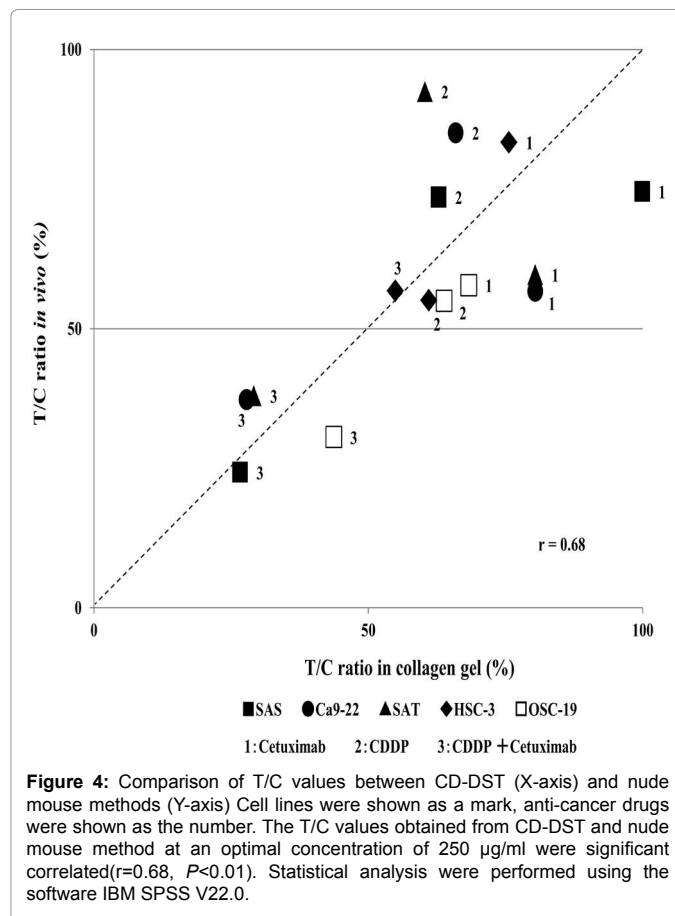
therapy group were lower than in the cetuximab monotherapy and CDDP+5-FU combination therapy groups.

Comparison of results from CD-DST and nude mouse studies

The chemosensitivity results from the nude mice methods are presented in Table 3. High sensitivity was defined as <50% *T/C*, whereas low sensitivity was defined as >50% *T/C*. The *T/C* values were largely consistent with the results obtained using the CD-DST method at an optimal contact concentration of 250 µg/ml (Figure 4). Antitumor effects tended to be significantly enhanced in the cetuximab+CDDP combination therapy group compared with the cetuximab and CDDP monotherapy groups (Figure 5; Bonferroni test; *P*<0.01)

Discussion

In recent years, there have been occasional reports on the use of chemosensitivity testing to select anticancer drugs for the treatment of individual tumors. Basic research publications have described the CD-DST evaluation method and chemotherapy approaches based on sensitivity testing for head and neck cancers [14]. The CD-DST method combines a 3D culture of single cells embedded in collagen droplets with image-based colorimetry. The main feature of this method is that testing can be performed even with a small sample because it uses a micro 3D culture with an extracellular matrix consisting of type I collagen gel, enabling high growth assay. CD-DST can also be used to evaluate the antitumor effects of anticancer drugs based on physiological drug concentration, whereas the adoption of an image analysis system can eliminate the effects of fibroblast contamination, making it possible to selectively evaluate the antitumor effects of medications directly on cancer cells. The technique has a success rate of at least 80% in colorectal, lung, breast, and other cancers [23-25] and has a 91% prediction rate

|	Ca9-22	SAS	SAT	HSC-3	OSC-19
CDDP	85.1	73.6	92.5	55.1	55
Cetuximab	56.7	74.6	59.7	83.4	57.8
CDDP+Cetuximab	38	38	37.3	56.8	30.6

Table 3: Results of nude mouse method (T/C values).
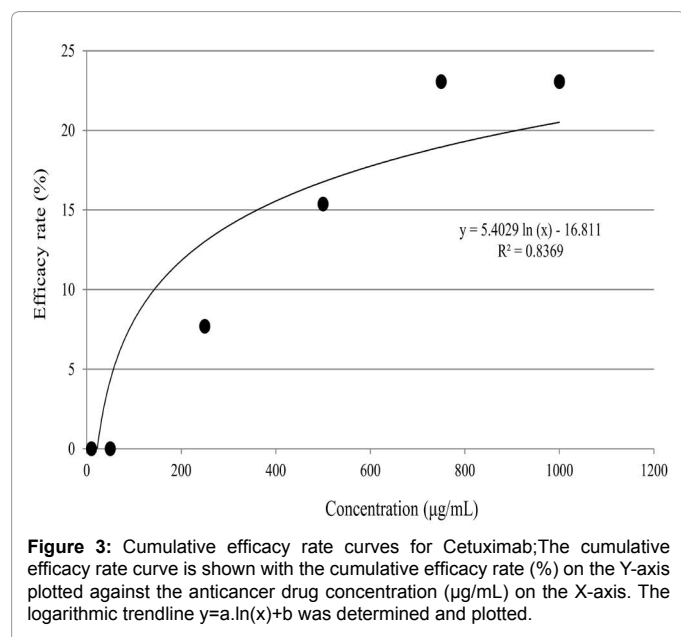
Figure 3: Cumulative efficacy rate curves for Cetuximab;The cumulative efficacy rate curve is shown with the cumulative efficacy rate (%) on the Y-axis plotted against the anticancer drug concentration (µg/mL) on the X-axis. The logarithmic trendline y=a.ln(x)+b was determined and plotted.

Figure 4: Comparison of T/C values between CD-DST (X-axis) and nude mouse methods (Y-axis) Cell lines were shown as a mark, anti-cancer drugs were shown as the number. The T/C values obtained from CD-DST and nude mouse method at an optimal concentration of 250 µg/ml were significant correlated(r=0.68, *P*<0.01). Statistical analysis were performed using the software IBM SPSS V22.0.

for clinical response [12]. This study evaluated its application to chemosensitivity testing for OSCCs.

The anti-EGFR antibody drug cetuximab has recently been used as a molecularly targeted therapy for recurrent and metastatic HNSCCs. A molecularly targeted therapy is a drug that targets various genes and proteins specific to cancer cells, such as cell surface antigens, growth factors and growth factor receptors, antiangiogenic factors, signaling cascades, and tumor suppressor genes [26,27]. Cetuximab competitively binds to overexpressed *EGFR* and blocks its signal transmission pathway [28], although it has also been reported to have an antitumor effect *via* modulation of the immunity of *EGFR*-expressing cells [29]. The discovery of specific biomarkers for resistant genes and antitumor effects has led to the development of personalized chemotherapy, the elimination of ineffective anticancer drug treatment, improved therapeutic responses, and reductions in patient physical and financial burden. However, there are currently no biomarkers for OSCC; therefore, personalized medicine based on molecularly targeted therapy is currently not available to patients with this type of cancer. Chemo-sensitivity testing of anticancer drugs is preferable to comprehensively predict the effects of cetuximab, and CD-DST is a suitable method for assessing its effects on OSCC due to the limited amount of tumor tissue. An optimal contact concentration needs to be set to predict cetuximab's antitumor effects using CD-DST, and it must also be possible to evaluate cetuximab in combination with other anticancer drugs.

In the present study, we sought to use CD-DST results to predict cetuximab antitumor effects by setting an optimal contact concentration
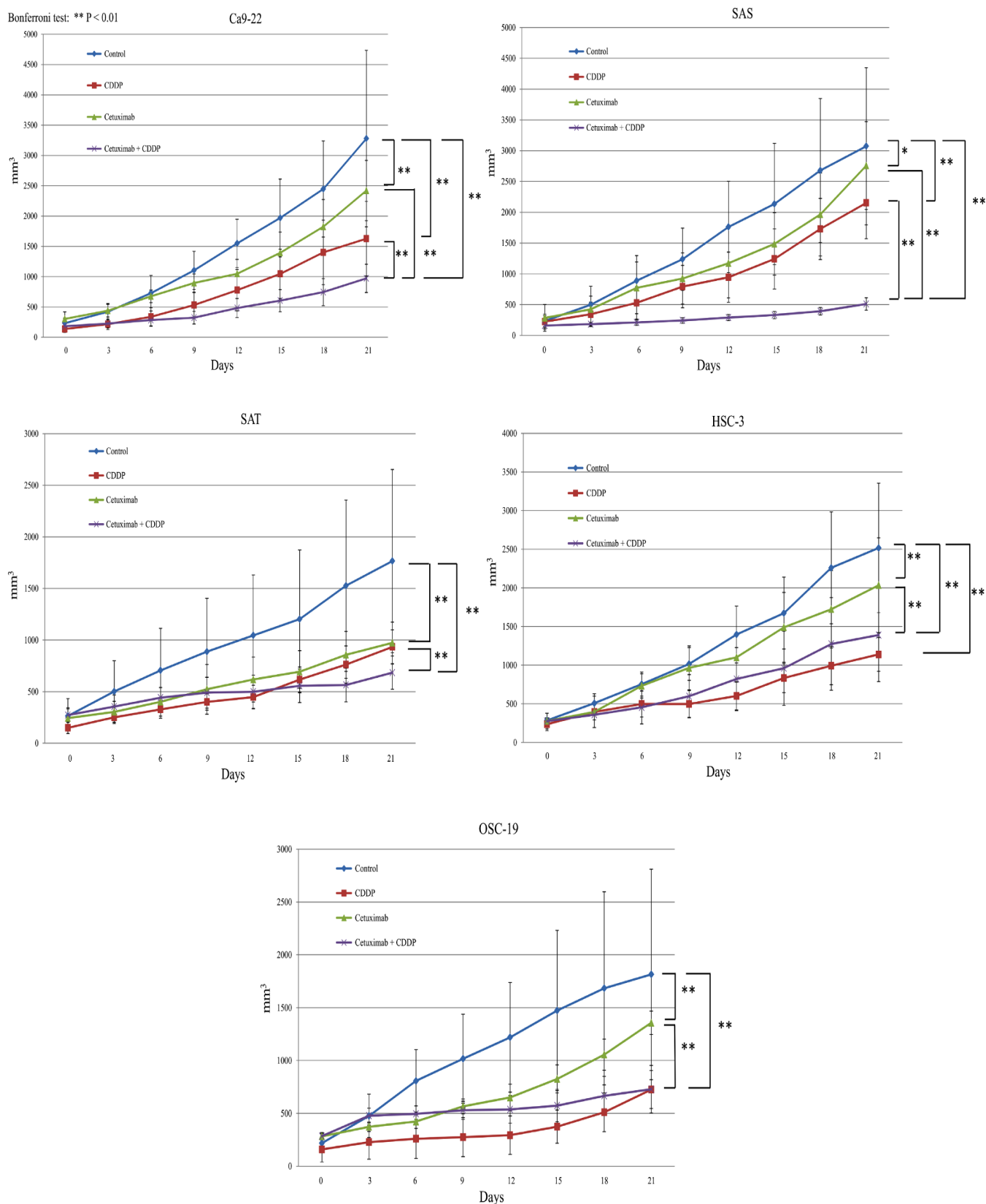
Figure 5: Antitumor effects of cetuximab+CDDP combination therapy in nude mice; Antitumor effects tended to be significantly enhanced in the cetuximab+CDDP combination therapy group compared with the cetuximab and CDDP monotherapy groups; Bonferroni test: Data are plotted as the mean ± SD (n=6). *P<0.05, **P<0.01.

and investigating how antitumor effect was enhanced when cetuximab was combined with cytotoxic anticancer drugs. Due to the small amount of OSCC sample remaining, we conducted an experiment using human cancer cell lines in place of clinical samples. We used PT-RCR to search for cetuximab-related genes expressed by each of these cell lines and investigated their respective specificities.

EGFR is overexpressed in more than 90% of OSCCs [30,31]. *HER2* and *HER3* are members of the *EGFR* family that possess potent tyrosine kinase activity and shows resistance to *EGFR*-targeted therapies [32,33]. However, *EGFRvIII* is a mutant form of *EGFR* that is expressed in 40% of head and neck cancers [34] and is believed to be involved in cetuximab resistance [35]. The tumor suppressor gene *PTEN* blocks the PI3K/AKT signal transmission pathway, and previous studies have suggested that the absence or diminished expression of PTEN contributes to cetuximab resistance [36,37].

EGFR was expressed in all 13 cell lines used in this study. In terms of *HER2, HER3, and EGFRvIII*, which tend to confer resistance *via* protein expression, the cell lines were positive for *HER3 and EGFRvIII* expression and were also positive for *PTEN*, which tends to confer resistance via its absence or reduced expression. The CD-DST results of cetuximab chemo-sensitivity testing alone using a nude mouse model showed low sensitivity, suggesting that it is difficult to predict cetuximab sensitivity in OSCC based on the various gene information identified in this study. No genetic uniqueness was observed in the 13 cell lines used in the present study. However, differences in drug susceptibility were observed among these cell lines, suggesting that other factors are involved. In particular, the cell line Nialym was clearly shown to have resistance, and the result warrants further studies in this cell line. The KRAS mutation, which is found in approximately 40% of whole colorectal cancer patients, was not included in search targets in the present study because it is rarely found in OSCC (6%) and the antitumor effect is difficult to predict with gene expression data.

We conducted a test to validate cetuximab antitumor effects at an estimated cetuximab concentration of 250 μg/ml compared with CDDP and CDDP+5-FU *in vivo* using the nude mouse method. Although the antitumor effects of *in vitro* testing were determined by drug concentration and duration of action, the plasma concentration of clinical doses is difficult to reproduce using the nude mouse method. To address this issue, Kondo et al. [19] used the LD_{50} [38] and maximum tolerated dose (MTD) of nude mice as the criterion for selecting a single intraperitoneal dose of 8 mg/kg CDDP as optimal, as this was the MTD at which no deaths occurred and at which animal weight loss was within 20%. After reducing the dose from the MTD and examining the change in plasma concentrations, Inaba et al. decided that a single intravenous dose of 7 mg/kg CDDP is an acceptable clinically equivalent dose [39,40]. Furthermore, Hara et al. [41] selected a single intraperitoneal dose of 8 mg/kg CDDP, which was similar to that reported by Inaba et al. In terms of cetuximab dosing. Luo et al. [20] used colon cancer GEO cells to measure cetuximab antitumor effects and active plasma concentrations after intraperitoneal injection of 0.04–0.25 mg cetuximab once every 3 days. Results indicated that a dose of 0.25 mg/3 days approached the plasma concentration observed in the clinic. We used these doses for nude mouse chemosensitivity testing and were able to reproduce clinical plasma concentrations for both CDDP and cetuximab, thus reflecting the antitumor response observed after clinical dosing. The results of CD-DST evaluation of cetuximab and CDDP alone and in combination were nearly the same as with the sensitivity observed in the five cell lines (Ca9-22, SAS, SAT, HSC-3, and OSC-19), essentially predicting the clinical effects of cetuximab.

ADCC activity is considered to be important for the antitumor effect of cetuximab, and it is desirable to include the antitumor effect from ADCC activity in the evaluation with CD-DST; however, it is difficult to perfectly reproduce *in vivo* effects in any *in vitro* anticancer drug susceptibility test. Nevertheless, the observed agreement in antitumor efficacy and susceptibility trend between CD-DST and the nude mouse method suggests the usefulness of CD-DST in clinical efficacy prediction and drug selection.

Cetuximab is one of a limited number of molecular targeted therapies that is effective against head and neck cancers, suppressing tumor cell proliferation, infiltration, and progression caused by the overexpression of *EGFR*; thus, it is expected to yield clinical utility in the future [42,43]. However, the inability of a recent clinical study to demonstrate significant enhancing effects for cetuximab in combination with CDDP chemotherapy of LA-SCCHN (RTOG0522 study) suggests that the use of cetuximab may not necessary improve therapeutic response. The CD-DST method is therefore very useful, as it enables evaluation of cetuximab sensitivity.

Although the putative mechanism by which cetuximab combination therapy enhances antitumor response has not yet been demonstrated, the findings from the present study suggest that CD-DST can contribute to the development of personalized chemotherapy by excluding ineffective anticancer drugs and maximizing therapeutic response to combination chemotherapy, including cetuximab. Chemotherapy has a major impact on patient's quality of life in terms of its physical and financial burden, and there is a strong argument for the use of CD-DST in chemosensitivity. It will therefore likely become an even more important testing method in future applications.

Conclusion

In conclusion, using the CD-DST method to test the sensitivity of 13 cell lines, we predicted that the optimal contact concentration of targeted molecular therapy cetuximab in the treatment of OSCC is 250 μg/ml. We then compared the CD-DST results for cetuximab monotherapy and cetuximab+CDDP combination therapy at this dose with the results from the nude mouse study and found that their respective sensitivities tended to be approximately equal. This finding suggested that CD-DST is a useful method for testing cetuximab sensitivity and that the selected contact concentration was valid. Among the anticancer drug groups, cell lines with low sensitivity tended to exhibit high sensitivity when the regimen was combined with cetuximab, suggesting that cetuximab may enhance the antitumor effects of existing cytotoxic anticancer drugs. In the future, chemosensitivity testing on clinical samples and comparing these results with patient therapeutic responses is required.

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