Identification of the optimal cetuximab concentration that is effective against oral squamous cell carcinoma in collagen gel droplet embedded culture drug sensitivity testing

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Abstract. Anticancer drug sensitivity testing using the collagen gel droplet embedded culture drug sensitivity test (CD-DST) on oral squamous cell carcinoma (OSCC) samples beginning from 2010 has been conducted. The present study investigated the effect of adding cetuximab (Erbitux®), a molecularly targeted drug, on anticancer drug activity against clinical OSCC specimens. A total of 25 specimens were obtained from 25 patients with OSCC between October 2013 and December 2017. The present study conducted anticancer drug sensitivity testing for cisplatin (CDDP), 5-fluorouracil (5-FU), cetuximab, three-drug combination, single agent and multi drug combinations, and cetuximab addition to the aforementioned regimens using CD-DST. In addition, the optimum concentration of each drug was evaluated. The overall evaluation success rate of the CD-DST method for OSCC specimens was 84.0% (21 of 25 cases); sensitivity to anticancer drugs and cetuximab could be evaluated. The *in vitro* efficacy rate of a cetuximab single agent and CDDP + 5-FU (PF) at a cut-off value of 50% was similar to the known clinical response rate. However, at a cut-off value of 50%, the *in vitro* efficacy of PF + cetuximab was calculated to be 40%, which was higher than the clinical response rate. The CD-DST method could be used to evaluate cetuximab, a molecularly targeted drug. Furthermore, its additive effect on conventional chemotherapy could be evaluated.

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The CD-DST method is suitable for evaluating and selecting chemotherapy regimens, including molecularly targeted drugs. Future studies are required to generate and evaluate relevant clinical data.

Introduction

Currently, there is no established methodology for the treatment of progressive, relapsed or metastatic in oral squamous cell carcinoma (OSCC). Cetuximab is a promising chemotherapeutic agent for treating OSCC that specifically binds to the epidermal growth factor receptor (EGFR). Cetuximab has been demonstrated to be associated with radiation therapy to improve local disease control rates and patient survival rates in locally advanced squamous cell carcinoma of the head and neck. In addition, treatment with cetuximab in conjunction with platinum drugs and fluorouracil was found to improve the survival of patients with recurrent or metastatic squamous cell carcinoma of head and neck (SCCHN) (1-4). However, there is no established predictor of treatment response in OSCC. Since the tumor response cannot be accurately predicted, patients are administered cetuximab regardless of tumor sensitivity. Moreover, cetuximab can result in infusion reactions in addition to other serious adverse events. Thus, the identification of predictors of treatment response in OSCC patients is essential to avoid ineffective drug administration and the risk associated with patients. Chemosensitivity tests used in the fields of colon cancer, stomach cancer and breast cancer are said to be effective for personalized medicine (5). In 1995, Kobayashi developed the CD-DST method, which is a chemical sensitivity test that combines a three-dimensional (3D) cell culture, serum-free culture, and an image-based colorimetric assay. The CD-DST method is used in many fields by overcoming many of the problems in chemical sensitivity tests that have been conducted so far (5,6). Since CD-DST uses a micro-3D culture, it can be used to evaluate activity against OSCCs, as well as other types of cancer with a low tumor volume. Further, its use in personalized medicine initiatives to evaluate cytotoxic anticancer drug sensitivity of head and neck cancer has been described (7). In this study, we investigated whether the effectiveness of sensitivity testing in clinical specimens

using cetuximab, a molecularly targeted drug, can be determined. The cut-off value of T/C% for assessing the efficacy of drug regimens including cisplatin (CDDP) and 5-fluorouracil (5-FU) in the presence or absence cetuximab in the CD-DST method was set to 40%.

Patients and methods

Patients. Twenty-five OSCC patients who consented to participate in the study from October 2013 to December 2017 in Nippon Dental University at Oral and Maxillofacial Surgery, Niigata Hospital (Table I). The Ethics Committee of The Nippon Dental University School of Life Dentistry at Niigata (Niigaka, Japan) approved the present study (approval no. ECNG-H-119).

CD-DST was performed on 25 primary lesions and one metastatic lymph node; 5-7-mm^2 tissues (approximately 0.25-0.5 mg) were collected mainly from the area surrounding the hardened part of the tumor. CD-DST was performed in accordance with the methods described by Kobayashi *et al* (6,8), using a human tumor cell primary culture system kit (Primaster®; Kurabo Industries Ltd., Osaka, Japan). Briefly, the samples were treated with Dispersion Enzyme Cocktail EZ (Primaster® reagent). The samples comprised of cell suspensions were transferred into collagen gel-coated flasks (CG flasks, a Primaster® device) and incubated overnight in pre-culture medium, PCM-1 (Primaster® content). The collagen gel was digested with EZ to obtain viable cancer cells. Type I collagen, F-12 medium 10x concentrate, and reconstitution buffer were mixed in ice water at a 8:1:1 ratio (Primaster® content). The cancer cell suspension (1×10^5 cells/ml) was then combined with the to the collagen solution. After 1 h, DF medium supplemented with 10% fetal bovine serum was added to each well; the plates were placed in a CO_2 incubator overnight at 37°C. The following final concentrations of the anticancer drugs were added: 0.5 μg/ml cisplatin (CDDP; Randa Injection 50 mg/100 ml; Nippon Kayaku, Tokyo, Japan) (9); and 0.7 μg/ml fluorouracil (5-FU injection 250 Kyowa®; Kyowa Hakko, Tokyo, Japan) (9). The cancer cells were incubated for 24 h. In addition, cetuximab (Erbitux® Injection 100 mg/20 ml; Merck Serono, Tokyo, Japan) was added at 250 μg/dl and incubated for 144 h as reported by Ryuki *et al* (10) (Table II). After removing the medium containing the 5-FU and CDDP anticancer agents, each well was washed twice with 3 ml of Hanks' Balanced Salt Solution and covered with 4 ml PCM-2 medium (Primaster® serum-free medium). When cetuximab was further added, it was incubated for 6 days after cetuximab addition. After the incubation, a final concentration of 50 μg/ml neutral red solution was added to each well. Surviving colonies in the collagen gel droplets were stained for 2 h and subsequently fixed in 10% neutral buffered formalin. After fixing, the samples rinsed with water and permitted to air-dry before being quantified by an optical density image analysis using Primage System® (Solution Systems, Tokyo, Japan) (Fig. 1). *In vitro* sensitivity was expressed as the T/C ratio of the optical density, where T represents the treated samples and C represents the controls; a T/C ratio of <50% was regarded as *in vitro* chemosensitivity. A tumor cell colony volume ratio (tumor growth rate) at 0 time was calculated using the control

Table I. Patient characteristics (n=25).

Characteristics	No. of patients (%)
Sex	
Male	15 (60.0)
Female	10 (40.0)
Age, mean, range	70.6, 37-93
Histology (Squamous cell carcinoma)	
Differentiation	19 (76.0)
Moderately differentiated	5 (20.0)
Poorly differentiated	1 (4.0)
Stage	
I	3 (1.2)
II	9 (36.0)
III	3 (12.0)
IV	10 (40.0)
Primary site	
Tongue	9 (36.0)
Buccal mucosa	6 (24.0)
Oral floor	2 (8.0)
Gingival	6 (24.0)
Hard palate	2 (8.0)
Resection mode	
Biopsy	20 (80.0)
Surgery	5 (20.0)
Sample site	
Neck metastasis	1 (4.0)
Primary	24 (96.0)
CD-DST	
Success	21 (84.0)
Failure	4 (16.0)

CD-DST, collagen gel droplet-embedded culture drug sensitivity test.

Table II. Anticancer agents and concentrations used in the CD-DST.

Agents	Concentration (μg/ml)	Time (h)
CDDP	0.5	24
5-FU	0.8	24
Cetuximab	250	144

CD-DST, collagen gel droplet-embedded culture drug sensitivity test.

group; a value of less than 0.8 was regarded as unsuccessful culture (low growth rate), regardless of the tumor cell colony volume in the control group.

Statistical analysis. Fisher's exact test was used to determine differences between groups. A value of P<0.05 was considered statistically significant (Ekuseru-Toukei 2015, Social Survey Research Information Co., Ltd.).
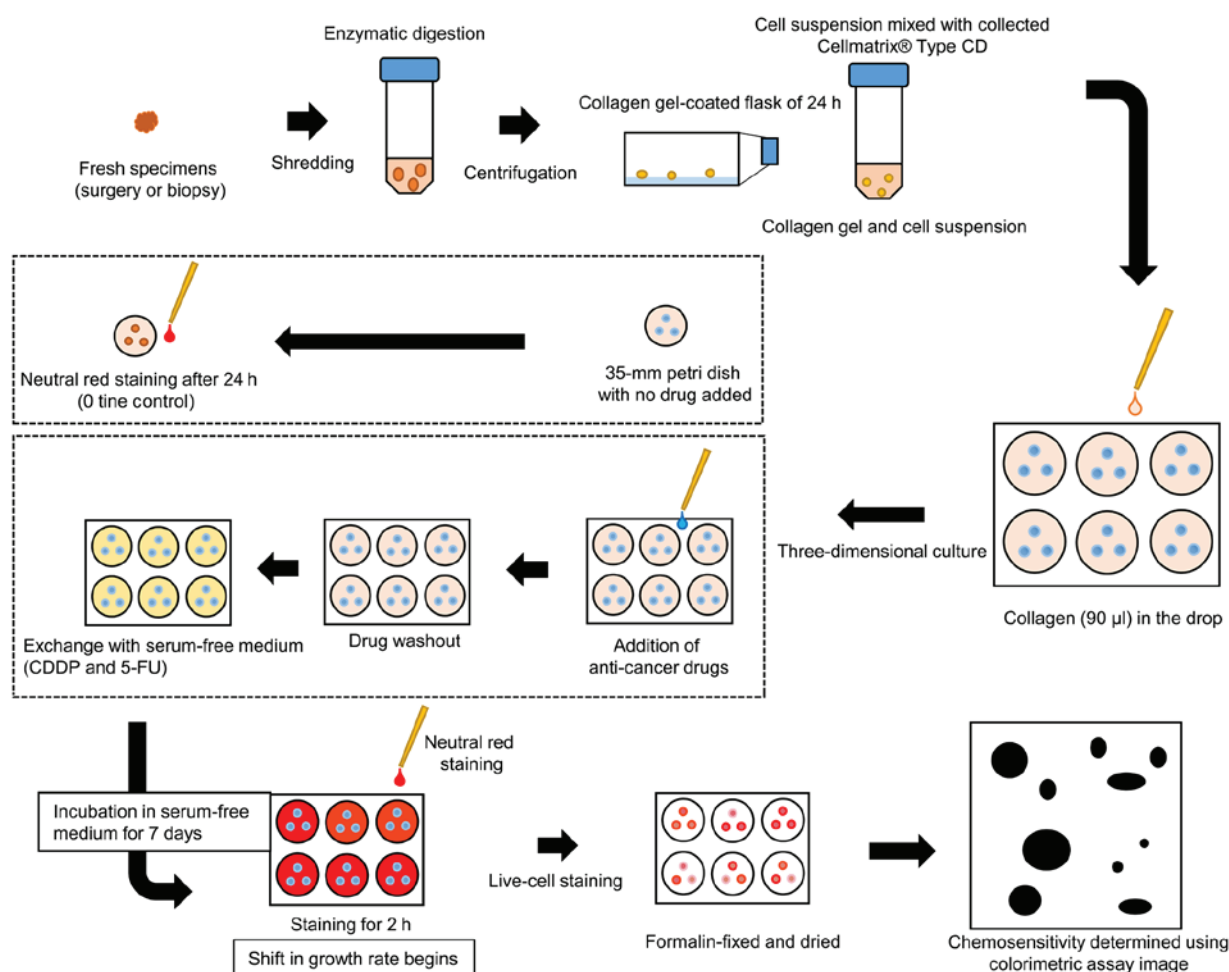
Figure 1. Overview of the collagen gel droplet embedded culture drug sensitivity test method. CDDP, cisplatin; 5-FU, 5-fluorouracil.

Results

Evaluation success rate using clinical specimens. CD-DST of cetuximab against OSCC had an overall evaluation success rate of 84.0% (21 of 25 cases), and could be applied to all anti-cancer agents used in the present study. The evaluation success rate was 83.3% (20 of 24 cases) for primary lesions and 100% (1 of 1 case) for metastatic lymph nodes (Table III). The causes of unsuccessful CD-DST were insufficient tumor cells (growth rate less than 0.8) in 2 cases and bacterial contamination in 2 cases. The mean tumor growth rate was 6.13±4.82. In addition, no significant difference was observed between sensitivity to various anticancer agents, stage, or histological differentiation (n.s.).

Comparison between in vitro efficacy rate and clinical response rate to various anticancer drugs. We compared the *in vitro* efficacy rate and clinical response rate of representative anticancer drugs used for clinical treatment of OSCC (2,11) (Table IV). PF's *in vitro* efficacy rate was 23.8%, which was similar to its clinical response rate of 20-30% (2,11). In addition, the *in vitro* efficacy rate of single agent cetuximab was 11.1%, which was similar to its clinical response rate of 13%. However, PF + cetuximab had an *in vitro* efficacy rate of 52.0% versus a clinical response rate of 36.0% (2).

Determination of cut-off value of PF + cetuximab in CD-DST. In anticancer drug sensitivity testing, conducted using the CD-DST method in the present study, the *in vitro* efficacy rate of PF + cetuximab was 52.3% at a cut-off value of 50%, which was higher than the clinical response rate. The cut-off value calculated with CD-DST data using PF + cetuximab in 21 samples and the clinical response rate (2) of PF + cetuximab, the cut-off value was 40%, which was similar to the clinical response rate of 33.3% (Fig. 2).

Discussion

Recently, the molecular targeting drug cetuximab has been used in combination with conventional anticancer agents for relapsed and metastatic SCCHN (12,13). Cetuximab competitively binds to EGFR expressed on the tumor and shows an antitumor effect by suppressing signal transduction (14,15). Specific biomarkers in anti-tumor effects have resulted in elimination of ineffective agents for chemotherapy, development of personalized chemotherapy, improvement of treatment response, and improvement of patients' QOL. However, biomarkers for cetuximab in the OSCC have not been defined. As a result, personalized therapy for molecular targeted therapies was not currently available to patients with OSCC. Therefore, predicting the therapeutic response of cetuximab in OSCC patients can avoid ineffective drug

Table III. Evaluation of the success rate of the CD-DST using oral squamous cell carcinoma clinical specimens.

Sample site	Number of samples	Evaluable cases	Evaluation success rate, %
Primary lesion	24	20	83.3 (20/24)[a]
Metastatic lymph node	1	1	100 (1/1)[a]
Total	25	21	84.0 (21/25)[a]

[a]Number of success cases/total number of assays. CD-DST, collagen gel droplet-embedded culture drug sensitivity test.

Table IV. *In vitro* sensitivity and clinical efficacy rates of individual drugs against oral squamous cell carcinoma.

Efficacy rate	PF	Cetuximab, 250 μg	PF + Cetuximab
CD-DST *in vitro* efficacy rate, %	23.8 (5/21)[a]	11.1 (2/18)[a]	52.3 (11/21)[a]
Clinical efficacy rate, %	20.0-30.0 (2,11)	13.0 (2)	36.0 (2)

[a]Number of effective cases/total number of assays. *In vitro* drug sensitivity was defined as positive when the T/C ratio was ≤50%. PF, cisplatin + 5-fluorouracil; CD-DST, collagen gel droplet-embedded culture drug sensitivity test.
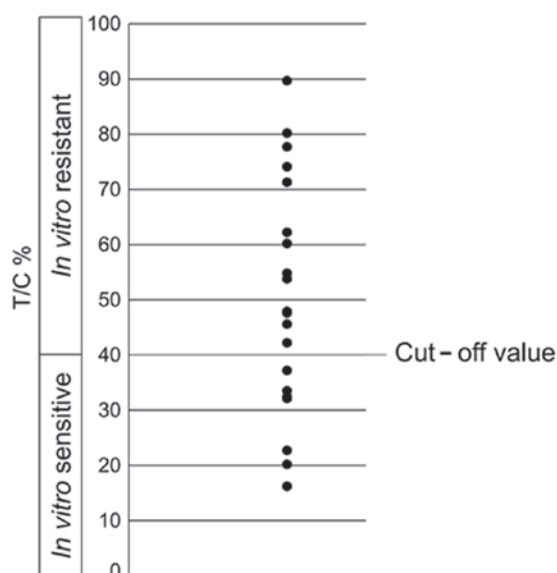

Figure 2. Determination of cut-off value of CDDP+5-FU+Cetuximab in CD-DST. In total, 21 cases who performed the CD-DST method with anticancer drug contact of CDDP 5-FU+Cetuximab are arranged in order of T/C%. The sensitivity of CD-DST for CDDP+5-FU+Cetuximab in oral cancer was found to be high when T/C% <40%, and lower when it was >40%. CD-DST, collagen gel droplet-embedded culture drug sensitivity test; CDDP, cisplatin; 5-FU, 5-fluorouracil; PF, CDDP+5-FU; T/C, total colony of the treated cells/total colony of the untreated cells.

administration and can avoid the patient adverse events associated therewith.

The CD-DST we performed in the present study has a potentially high success rate, based on initial culture, needs a small number of cells, and uses serum-free culture medium and image colorimetry to eliminate the influence of fibroblasts. The CD-DST has the advantage of using an anticancer drug concentration comparable to the clinical therapeutic dose (5,6,9,10).

In fact, the primary measurement success rate of the CD-DST was reported to be 87.5% in colorectal cancer, 79.2% in lung cancer, and 84.3% in breast cancer (16-18), and it shows a high clinical efficacy prediction rate of 91% (5). Similarly, in sensitivity testing using cisplatin, fluorouracil, or docetaxel in OSCC, as we reported earlier, the primary culture success rate was 81.8% (19). In the present study, we conducted anticancer drug sensitivity testing, including cetuximab specimens of OSCC cases, with a success rate of 83.3% (20 of 25 cases) in primary tumors, 100% (1 out of 1 case) in metastatic lymph nodes, and 84.0% overall (21 of 25 cases). This result suggested that the CD-DST method could also evaluate anticancer drugs including cetuximab in OSCC.

Kobayashi *et al* (5,6) reported a statistically significant correlation between *in vitro* efficacy in CD-DST and clinical response of breast cancer, gastric cancer, colorectal cancer, and lung cancer. In the present study on OSCC, the 23.8% *in vitro* efficacy rate of PF therapy was comparable to the previously reported 20.0-30.0% clinical response rate to preoperative PF chemotherapy (2,11). Further, the 11.1% *in vitro* efficacy rate of cetuximab therapy in the present study was comparable to the reported clinical response rate of 13.0% (2). Ryuki *et al* (10) reported a concentration of cetuximab alone in the CD-DST method using a human oral cancer cell line of 250 μg/ml. We used cetuximab alone at 250 μg/ml based on the above study. We found the *in vitro* efficacy was consistent with the clinical response rate. However, when the CD-DST method was performed with PF + cetuximab (250 μg/ml), the *in vitro* response rate was 52.3%, which was higher than the clinical response rate of 36.0%. The *in vitro* efficacy rate after changing the cut-off value from 50 to 40% was 33.3% (7 out of 21), similar to the clinical response rate of 36.0%. The sensitivity of CD-DST for PF + cetuximab in oral cancer was found to be high when T/C% was less than 40%, and lower when it was more than 40%.

We previously performed CD-DST in a recurrent postoperative course of hard palate cancer (T2N2 cM0: Stage IVA)

and compared the results to those of sensitivity testing of PF + cetuximab and its clinical efficacy (20). In the same study, the T/C% of the CD-DST method was found to represent high sensitivity at 32.4%, and the clinical efficacy of PF + cetuximab was consistent with the partial response (20). The reported T/C% is 32.4%, which is less than 40% of the cut-off value we determined in the present study, and can be considered to represent high sensitivity. Further, because it is consistent with clinical efficacy, the cut-off value of 40% determined in the present study appears appropriate. In addition, the antitumor effects of cetuximab include antibody-dependent cell-mediated cytotoxicity (ADCC) activity in addition to its inhibitory effect on EGFR signaling (21). Although it is challenging to evaluate ADCC activity *in vitro*, a comprehensive evaluation was possible in the present study because the drug effect was assessed based on results of CD-DST and clinical response rate.

The mechanism by which the antitumor effect is enhanced has not been demonstrated when cetuximab is used in combination with conventional anticancer agents. It has been suggested that the CD-DST method can contribute to the development of individualized chemotherapy by predicting the treatment effect when the anticancer drug is administered with cetuximab in the upper part. Cetuximab alone and the effect of adding cetuximab to combination therapy can be evaluated.

Chemotherapy, including molecularly targeted drugs, can increase the physical and economic burden on patients and affect their quality of life. Therefore, it was suggested that the application of CD-DST method could become an increasingly important test method in the future.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KS and SH have made substantial contributions to the conception and design of the study, and the acquisition, analysis and interpretation of data. HT and AT were involved in drafting the manuscript and critically revised it for important intellectual content. HT and AT made substantial contributions to the conception and design of the study, and the acquisition, analysis and interpretation of data.

Ethics approval and consent to participate

The patients consented to participate in the study. The Ethics Committee of The Nippon Dental University School of Life Dentistry at Niigata (Niigata, China) approved the present study (approval no. ECNG-H-119).

Patient consent for publication

Written informed consent was obtained from the patients.

Competing interests

The authors declare that they have no competing interests.

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