Histoculture Drug Response Assay for Identifying Effective Anticancer Agents for Oral Squamous Cell Carcinoma: Report of Two Cases

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Abstract

Two cases of unresectable squamous cell carcinoma of the oral cavity were treated with combination chemotherapy using drugs determined to be effective in histoculture drug response assay with MTT end point. Both cases showed good response to the chemotherapy. The present results suggest that chemosensitivity testing facilitates individualization of chemotherapy.

Introduction

Treatments for oral carcinoma include surgery, radiotherapy, chemotherapy and combination therapy. Many anticancer drugs have been developed for clinical use, and their clinical response rates have been assessed (1-3). In general, the combination of cisplatin (CDDP) and 5-fluorouracil (5-FU) has been widely used, and is generally considered the first choice for oral squamous cell carcinoma (SCC)(4). However, some cases do not respond to this combined chemotherapy, and it is difficult to determine the appropriate drug for each patient or predict the response to chemotherapy.

Yamamoto et al. (5) reported that mode of invasion correlated well with bleomycin (BLM) sensitivity of oral SCC. Many *in vivo* and *in vitro* chemosensitivity tests for anticancer drugs have been developed to predict response of tumors to chemotherapeutic agents in individual patients (6). Histoculture drug response assay (HDRA) is based on a tissue culture system that involves a popular method of tumor culture. Mosmann (7) reported that the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] assay is a rapid, cheap, and convenient colorimetric assay of cellular activity *in vitro*.

We recently used HDRA with assessment by MTT end point to predict sensitivity of anticancer agents. Here, we report two cases of advanced SCC of the lower gingiva that responded well to the anticancer agents selected according to the results of the assay.

Methods

HDRA with MTT end point

Cancer specimens were obtained under local anesthesia along with biopsy specimens, and a portion of each was sent to the Department of General and Gastroenterological Surgery Laboratory, Osaka Medical College, where they were subjected to HDRA with an MTT end point. Tissues were explanted using a three-dimensional *in vitro* histoculture system developed by Hoffman et al. (8). Specialized aseptic collagen gels were cut with scissors into 1-cm³ pieces and placed into 24-well plates, after which cancerous portions of the specimens were minced using scissors, and were then placed on each of the prepared collagen surfaces. Anticancer drugs were dissolved at various concentrations, as indicated below, in RPMI-1640 medium containing 20% fetal calf serum, and 1 ml of the solution was added to each well; it reached the upper part of the gel, without covering the top. The plates were then incubated for 7 days at 37°C in a humidified atmosphere containing 95% air and 5% CO₂.

After histoculture, a collagenase and MTT solution, prepared by dissolving the solid portion in phosphate-buffered saline (PBS), was added to each culture well, followed by incubation for another 8 hours. Collagen gels were dissolved and the tumor pieces, which by then were floating in the medium, became violet colored. The medium was aspirated completely from each well, and dimethyl sulfoxide (DMSO) was added to dissolve the MTT-formazan product. The absorbance of the solution in each well was read at 540 nm using an ELISA reader. Absorbance per gram

was calculated, and the inhibition rate was calculated as follows: inhibition rate (%) = (1 - mean absorbance per g of tumor specimen in the treated wells / mean absorbance per g of tumor specimen in the control wells) × 100. When the inhibition rate was ≥50%, the chemosensitivity of the specimen to a particular drug was scored as positive.

The final concentrations of anticancer drugs used in this study were 300 µg/ml for 5-FU, 20 µg/ml for CDDP, 4.6 µg/ml for 4-O-tetrahydropyranyl adriamycin (THP) (10 times the peak plasma concentration), and 20 µg/ml for BLM (10 times the peak plasma concentration) (9,10).

Case Reports

Case 1

A 53-year-old Japanese man was referred to our department because of slow healing of an extraction socket in the left lower molar region over a 4-month period and paresthesia in the left lower lip. The only apparent abnormality observed in an extraoral examination was a left submandibular lymphadenopathy. An intraoral examination revealed a granulomatous exophytic tumor mass extending from the left lower premolar area to the retromolar region (Fig. 1). Dynamic contrast enhanced MRI showed that the tumor mass was relatively well defined, with a rapid enhancement and gradual wash-out pattern (11) (Fig. 2). A clinical diagnosis of gingival carcinoma (T4N1M0) was made. An incisional biopsy was performed under local anesthesia, and a well-differentiated SCC was

Fig. 1. Intraoral view at the first visit (case 1)
A granulomatous exophytic tumor mass extending from the left lower premolar area to the retromolar region was found.

Fig. 2. Dynamic contrast enhanced MRI (case 1)
The tumor was delineated as relatively well-defined mass with a rapid enhancement and gradual wash-out enhancement pattern.
Top left: pre contrast enhancement. Top center-bottom center: early phase image. Bottom right: delayed image.

histopathologically diagnosed (Fig. 3). Part of the specimen was subjected to HDRA with an MTT end point. The results of a chemosensitivity test indicated that cultures were sensitive to 5-FU, CDDP, THP and BLM (Table 1). Systemic chemotherapy consisting of intramuscular injection of BLM (total dose, 80 mg) and intravenous injections of THP (total dose, 50 mg), CDDP (total dose, 60 mg) and 5-FU (total dose, 4500 mg) was performed. Clinically, the tumor apparently decreased in size. After the patient made a general recovery, radiotherapy (total dose, 60 Gy) and intraarterial chemotherapy (BLM, 45 mg; CDDP, 30 mg) were performed in combination, after which we clinically diagnosed a complete response (Fig. 4). However, 4 months after completion of chemo-radiotherapy, tumor recurrence was observed. A radical operation consisting of a left hemimandibulectomy and hemiglossectomy combined with a left supraomohyoid neck dissection, followed by immediate reconstruction using a free scapular bone flap, was performed under general anesthesia. Local recurrence of the tumor was again observed, and the patient died 8 months after the operation.

Case 2

A 56-year-old Japanese woman was referred to our department with a bulky mass that had perforated the skin surface of her right mandible. The mass was a firm tumor extending from the right submandibular to mandibular region. Part of the skin overlying the mass had been

Fig. 3. Photomicrograph of incisional biopsy (case 1)
Original magnification $\times 250$, Hematoxylin-Eosin stain

Fig. 4. Intraoral view after combined chemo-radiotherapy (case 1)
Tumor mass had apparently decreased in size, and diagnosis was complete response.

perforated, with a sinus tract continuing to the oral cavity. Paresthesia of the right lower lip was also noted. An intraoral examination revealed a granulomatous tumor mass extending from the right lower canine to the ipsilateral retromolar area (Fig. 5). Dynamic contrast enhanced MRI showed a large relatively well-defined mass lesion with a short time to peak and gradual wash-out pattern (Fig. 6). A clinical diagnosis of gingival cancer (T4N1M0) was made. An incisional biopsy and HDRA with an MTT end point were performed, and the histopathological diagnosis was well-differentiated SCC (Fig. 7). Results of a chemosensitivity test indicated that cultures were sensitive to 5-FU, CDDP and BLM (Table 1). We administered

combination chemotherapy consisting of intramuscular injection of BLM (total dose, 40 mg), followed by intravenous injection of CDDP (80 mg) and 5-FU (total dose, 3750 mg). The tumor decreased in size, but the patient suffered from severe diarrhea after the combined chemotherapy. An additional 2 courses of intravenous chemotherapy consisted of Docetaxel (TXT, 80 mg) and Nedaplatin (CDGP, 110 mg), and were followed by irradiation (total dose, 65 Gy). After this combined chemo-radiotherapy, we diagnosed the clinical response as partial response. Uncontrollable tumor re-growth was observed, and the patient died 9 months after completion of the combined chemo-radiotherapy.

Fig. 5. Extra- and intraoral view at the first visit (case 2)
The skin overlying the mass was perforated, with a sinus tract continuing to the oral cavity. Intraorally, a granulomatous tumor mass extended from the right lower canine to the ipsilateral retromolar area.

Table 1. Calculated inhibition rate against each agent

Case No.	5-FU	CDDP	THP	BLM
1	75.1%	72.1%	78.5%	50.6%
2	51.9%	87.5%	46.5%	80.6%

Fig. 6. Dynamic contrast enhanced MRI (case 2)

A relatively well-defined large mass lesion with a short time to peak and a gradual wash-out pattern was observed.

Top left: pre contrast enhancement. Top center-bottom center: early phase image. Bottom right: delayed image.

Fig. 7. Photomicrograph of incisional biopsy (case 2)
Original magnification $\times 250$, Hematoxylin-Eosin stain

Discussion

The ability to predict chemosensitivity to anticancer drugs before starting chemotherapy is essential for individualization of chemotherapy. Patients with tumors of similar histopathological classification can have markedly different clinical drug-response spectra. In oral SCC, the

mode of invasion (a histopathological parameter) correlates well with tumor aggressiveness, metastasis, outcome and chemosensitivity (5). In the past few decades, there have been many studies of *in vitro* methods for accurately predicting the *in vivo* chemosensitivity of human tumors. The three-dimensional histoculture assay developed by Hoffman et al. allows fresh surgical specimens to maintain their cell-to-cell contact and three-dimensional native tissue architecture in culture (8). The MTT end point is a simple colorimetric test of cell proliferation and survival (7). In our institution, we use the results of HDRA with an MTT end point in selection of anticancer agents.

Cortazar et al. (12) reviewed the efficacy of an *in vitro* drug sensitivity test using computer searches, and pointed out that one potential limitation to the ability to select chemotherapy by *in vitro* drug sensitivity test involves the acquisition of tumor tissue. With the exception of advanced carcinomas such as those in the present cases, oral carcinomas are relatively small, making it difficult to obtain sufficient quantity of specimen for both chemosensitivity testing and histopathological examination. We believe that, for relatively early stage carcinoma diagnosed by excisional biopsy, a histopathological examination (which provides an abundance of information about the tumor) must take precedence over a chemosensitivity test. However, for a locally advanced tumor large enough to provide specimens for both examinations (as in the present cases), we believe that a chemosensitivity test can provide information useful in deciding which anticancer drugs are appropriate for the patient. Many cases of advanced inoperable oral carcinoma are complicated with other systemic diseases, and tend to show poor general condition; in such cases, a chemosensitivity test is useful because it can exclude drugs that do not induce anticancer effects but only cause severe adverse effects.

Several studies of chemosensitivity tests for head and neck malignancies have been reported. Nakashima et al. (13) reported that head and neck SCC was more sensitive to certain drugs than gastric cancer. Also, they found that inhibition of succinate dehydrogenase (SD) activity by anticancer drugs was always stronger in cancer cells originating in metastatic lymph nodes than in the primary tumor. They also reported that the histopathologic degree of differentiation of SCC affected SD inhibition: poorly

differentiated SCC is more sensitive to anticancer drugs than well-differentiated SCC (14). These findings, excluding the comparison between head and neck SCC and gastric cancer, differ from the present clinical findings. Robbins et al. (15) reported that sponge-gel-supported HDRA of the head and neck correlated well with clinical response, and discussed the importance of three-dimensionality of cultures. Both of the present cases were diagnosed as well-differentiated SCC, and the results of the chemosensitivity tests indicated that the tumors were sensitive to anticancer drugs. Clinically, good responses were obtained using drugs selected according to the results of the chemosensitivity test. However, further clinical trials are needed to examine correlation between clinical response, clinico-pathologic features, and chemosensitivity test results.

Kubota et al. (16) found that the HDRA response correlated with patient survival, suggesting that the HDRA can contribute to survival of gastric cancer patients when used prospectively. They also discussed the importance of a three-dimensional tumor culture for obtaining accurate clinical information. Singh et al. (17) recently reported that chemosensitivity determined by the HDRA appeared to be a strong predictor of survival in patients with advanced head and neck SCC. In the two present cases, although the results of HDRA correlated well with clinical response, they did not predict patient survival. Clinically, many factors influence patient survival, including tumor site, growth pattern, tumor stage and histopathological degree of differentiation. Further studies are needed to clarify relationships between these factors.

Tanigawa et al. (18) reported that tumor angiogenesis is one of the parameters that predict hematogenous metastasis (but not peritoneal or lymph node metastasis) and prognosis in patients with colorectal cancer. Moriyama et al. (19) reported that the mode of invasion of oral carcinoma correlated well with vessel density, which was calculated by counting the number of vessels stained with a mouse anti-human vascular endothelial cell antibody, JC-70A. In their examination of the relationship between vessel density and mode of cancer invasion, vessel density of tumors with a well-defined borderline mode of invasion was significantly higher than vessel density of carcinomas with other modes of invasion. They also noted that when predicting metastasis and establishing a prognosis, not only angiogenesis but also

lymphatic vessel neogenesis should be further examined. In addition, it is well accepted that one of the clinico-pathologic features that influence drug sensitivity is mode of invasion; carcinomas with a well-defined borderline are more strongly affected by BLM, and good clinical courses have been achieved (5). Recently, Hawighorst et al. (20) reported a relationship between standard and pharmacokinetic analyses of time-intensity curves from dynamic MRI, and reported histomorphological markers of tumor angiogenesis in uterine cervical carcinoma. We recently encountered tongue squamous cell carcinoma with a well-defined borderline in contrast enhanced MRI, which tended to show a well defined borderline in histopathologic examination (21). In the present cases, dynamic contrast enhanced MRI showed a rapidly and markedly enhanced pattern, and the borderlines were relatively well defined. These features suggest that both tumors were well vascularized, with a microscopically well-defined borderline, meaning that intravenously injected anticancer drugs would reach deep into the tumor tissues. Thus, we believe that a combination of contrast enhanced MRI and chemosensitivity test can predict the chemosensitivity of individual patients.

The present findings indicate that three main points should be considered when determining the utility of HDRA with an MTT end point in oral SCC: 1) tumor size, as indicated by HDRA; 2) the relationship between elements of clinico-pathologic examination (including imaging modalities) and chemosensitivity tests; 3) the relationship between patient outcome and chemosensitivity test results. Further study is needed to determine the reliability of these findings and the usefulness of HDRA.

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