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In vitro chemosensitivity profile of oral squamous cell cancer and its correlation with clinical response to chemotherapy

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Abstract

CONTEXT: Oral cancers represent a disparate group of tumors with diverse clinical behavior and chemosensitivity profile. Currently, it is difficult to predict whether a tumor will respond to chemotherapy and which drug(s) will achieve the maximum clinical response. **AIMS:** To study *in vitro* chemosensitivity profile of oral cancers and to correlate the *in vitro* chemosensitivity of oral cancer to clinical response to chemotherapy. **SETTINGS AND DESIGN:** Prospective study in a tertiary cancer care center. **METHODS AND MATERIAL:** We prospectively studied the chemosensitivity profile of 57 untreated, advanced, unresectable oral cancers to cisplatin, methotrexate, 5-fluorouracil and their combinations by using histoculture drug response assay (HDRA) and correlated them to the clinical response to chemotherapy. **STATISTICAL ANALYSIS USED:** *Chi Square* test. **RESULTS:** Biopsy samples were successfully histocultured in 52/57 (91%) cases. Of these 52 evaluable patients, 47 had primary gingivo-buccal cancers and five had tongue / floor of mouth cancers. Based on the assay, 27 (52%) tumors were sensitive to cisplatin, 27 (52%) to methotrexate, 24 (46%) to 5-fluorouracil, 38 (73%) to combination of cisplatin and methotrexate and 36 (69%) to combination of cisplatin and 5-fluorouracil. Of these, 31 patients with good performance status received two cycles of chemotherapy using one or more of these test drugs. There was a significant correlation ($p=0.03$) between the *in vitro* chemosensitivity and the clinical response. Negative predictive value of the test was 80%, positive predictive value-69%, sensitivity-79% and specificity -71%. The overall accuracy of the assay was 74%. **CONCLUSIONS:** We found HDRA to be a fairly good predictor of chemo-response of oral cancer.

Key words: Assay, chemotherapy, cisplatin, fluorouracil, methotrexate, neoplasm oral, response.

Introduction

Oral cancers represent a disparate group of tumors with diverse clinical behavior. Surgery, in association with radiotherapy, is the mainstay of treatment for advanced oral cancers. Results of chemotherapy alone in advanced head and neck cancers per se have not been very promising but induction and concomitant chemotherapy do have a role in organ preservation.^[1,2] Chemotherapy, in neoadjuvant setting as well as in recurrent disease, is known to decrease incidence of distant metastasis and delay it.^[3] Further, the response

to chemotherapy is intricately related with the response to radiotherapy.^[4] At present, it is not possible to envisage in advance whether a tumor will respond to chemotherapy and which drug(s) will be most effective. If we manage to predict the chemo-response of tumors before the initiation of chemotherapy, we can select the responders and optimal chemotherapeutic regimen for them, thereby deriving maximum benefit. Unnecessary

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chemo-toxicities in the non-responders can be avoided.

Histoculture drug response assay (HDRA) is a three dimensional native state histoculture assay that simulates the structure of tumor in the body and is used to assay the chemo-responsiveness of the tumor.^[5] This technique has been employed earlier in gastro-intestinal,^[6] ovarian,^[7] uro-genital^[8] and breast cancers^[9] with excellent results. There are only a few studies in English literature on application of this technique in head and neck squamous cell carcinoma^[10-14] and only the last one deals solely with oral cancer. Our study is unique as the *in vitro* efficacy of all the test drugs and their combinations are tested on the same tumors and clinical response of oral cancer to a particular drug was correlated with its *in vitro* chemosensitivity, as shown by HDRA.

Materials and Methods

Prior to accrual on the protocol, all 57 previously untreated patients with advanced, unresectable oral cancer (stage IVB) signed an informed consent approved by the Ethics Committee of the hospital. Biopsy specimen from their tumors were obtained in the outpatient clinic and transported to the laboratory in Hanks' balanced salt solution (HBSS; GIBCO). HDRA was performed as described by Furukawa *et al.*^[15] with slight modifications.

Technique

Histoculture was performed in triplicate with negative control, each drug and the combinations thereof. The collagen gel sponge (Gel Foam- Pharmacia and Upjohn Inc, USA) was cut into 1 cm³ pieces and placed into the wells containing RPMI 1640 medium (Sigma). Biopsy specimens were dissected and grossly viable tumor, free of fibrous connective tissue, was cut into approximately 10-mg pieces, weighted by chemical balance and placed onto these collagen gel sponges. After 24h, all the plates were examined for viability and any infection before RPMI 1640 medium containing test drug(s) replaced the original solution. Based on plasma drug levels and validation studies in our laboratory, the concentration used were 20 µg/ml of cisplatin (CDDP), 25 µg/ml of 5-Fluorouracil (5-FU) and 25 µg/ml of Methotrexate (MTX). Anticancer drugs were individually dissolved in RPMI 1640 medium (Sigma) containing 20% fetal calf serum, penicillin-streptomycin-amphotericin B (100 µu/ml, 100 mg/ml and 0.25 µg/ml, respectively) and 1 ml of the solution was poured per well into 24 wells plate. Subsequently, they were cultured in 5% CO₂ incubator at 37°C for seven days and thereafter 100 µl of 0.06% collagenase type I (Sigma) solution

in HBSS and 100 µl of 0.2% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (Sigma) in phosphate buffer saline (PBS) solution, containing 50 mM sodium succinate, was added to each well. The plates were incubated again for 16 hours, the medium was removed and 0.5 ml of dimethyl sulfoxide (DMSO) was added into the each well to extract MTT-formazan. After two hours, 100 µl of extract solution of each was moved to 96-well plate. The absorbance was measured at 540 nm and the inhibition rate was calculated using the formula; Inhibition Rate (I.R.) (%) = (1 - A/B) x 100, where A is a mean absorbance of the treatment wells per gram tumor and B is a mean absorbance of the control wells per gram tumor. An inhibition rate of over 50% was considered as an indicator of chemosensitivity as reported earlier.^[6,15] Tumors with inhibition rate of less than 50% were considered chemo-resistant. Only those samples, that did not show any infection in any of the wells, were analyzed provided their results were evaluable for the entire set (all the drugs and their combinations). The clinician evaluating the response to chemotherapy was blinded to the *in vitro* inhibition rate.

Chemotherapy

Thirty-one patients, with good performance status (ECOG/Zubrod score of 0 or 1), normal hematological and biochemical parameters, received two cycles of cisplatin based chemotherapy. Based on their chemosensitivity profile, nine (29%) patients received cisplatin (100 mg/m²) on day one with 5FU (1000 mg/m²) on days one to five administered over 120h by continuous infusion along with leucovorin (30 mg/day) repeated every three weekly; 9(29%) received cisplatin (100 mg/m²) on day one with methotrexate (50 mg/m²) on days one and eight repeated every three weekly and 12(39%) received three weekly cisplatin (100 mg/m²) alone. One patient received a combination of cisplatin, paclitaxel and ifosfamide (PIP regime). Clinical response was assessed two weeks after completion of two cycles of chemotherapy by a clinician who was blinded to the *in vitro* inhibition results. Patients were categorized as clinical non-responders (disease progression or no response to chemotherapy) or clinical responders (complete response or partial response to chemotherapy). The clinical response was correlated by using Chi-square test with the *in vitro* chemosensitivity assessed independently prior to initiation of chemotherapy. Sensitivity, specificity, predictive values and accuracy of HDRA were calculated.

Results

Out of 57 tumors, 52 (91%) could be successfully histocultured. Initial five patients (9%), who had

bacterial contamination in any of the wells, were excluded from further analyses. Data of 52 patients (42 males and 10 females) in the age range (47 ± 13) years were analyzed. Gingivo-buccal complex was the most common site of primary tumor in 47 patients (90%) and the rest five (10%) had tumor in the tongue or floor of mouth. All these patients were evaluated in multidisciplinary clinic and had unresectable T4 primary tumors which were not suitable for any radical loco-regional treatment. Of these, nine patients (17%) had node negative neck, 22(42%) had N1 disease, 18(35%) had N2 and three (6%) had N3 disease.

Figure 1 shows the inhibition rate of all samples on incubation with different chemotherapeutic agents and their combinations. The inhibition rates of 52 evaluable tumors with different drugs and combinations are summarized in Table 1. Taking a cut off inhibition rate of 50% as an indicator of chemosensitivity 46(88%) tumors showed sensitivity to one or more drug(s) with 27 (52%) tumors sensitive to cisplatin, 27 (52%) to methotrexate and 24 (46%) to 5-fluorouracil. Combination of chemotherapeutic agents led to a greater extent of inhibition than the individual drugs alone as 38 (73%) tumors were sensitive to a combination of cisplatin and methotrexate and 36 (69%) were sensitive to a combination of cisplatin and 5-FU.

Of these 52 patients, 31 with good performance status for chemotherapy, underwent two cycles of chemotherapy prior to evaluation of clinical response. Only one (3%) patient had complete clinical response and he subsequently received radical chemo-radiation. Of 13(42%) patients who had partial response, two down-staged tumors underwent radical excision followed

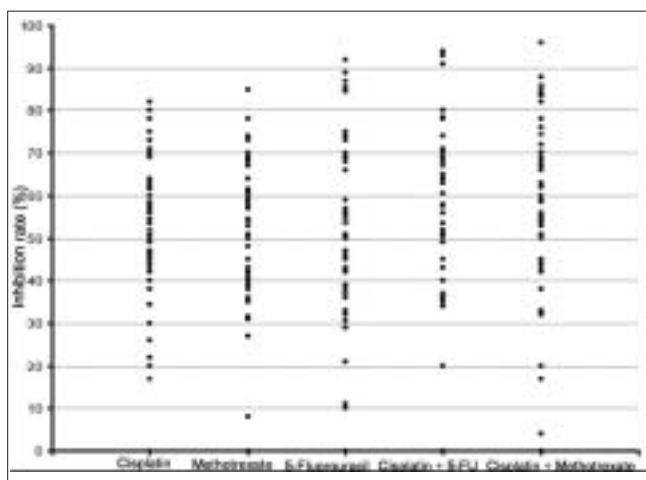


Figure 1: Chemosensitivity of oral cancer to different chemotherapeutic agents

by adjuvant chemo-radiation and the rest eleven underwent palliative radiotherapy. The remaining 17(55%) patients had no clinical response or disease progression. As the objective of this study was only to correlate clinical response to *in vitro* chemosensitivity, survival analysis was not performed on these patients. There was a significant correlation ($P=0.03$) between the *in vitro* chemosensitivity and the clinical response to chemotherapy. *In vitro* chemosensitivity, as predicted by HDRA, had a sensitivity of 79% and specificity of 71%. Positive predictive value of the test was 69% and negative predictive value was 80%. The overall accuracy of the assay was 74%.

Discussion

Surgery and radiotherapy with or without concurrent chemotherapy form the mainstay of management of advanced oral cancers. There is no unanimity in the literature regarding the role of neoadjuvant chemotherapy prior to loco-regional treatment with some uncontrolled trials showing a survival advantage^[16,17] and others showing no advantage^[18] or a detriment.^[19] Two randomized controlled trials have shown a trend towards decreased survival in neoadjuvant chemotherapy arm as compared to the standard arm but the small size of both these trial did not show any statistically significant difference.^[20,21] In a meta-analysis on the timing of chemotherapy, induction chemotherapy offered only a non-significant 2% survival advantage both at two and five years.^[22] Although chemo-responsiveness, per se, is an indicator of better prognosis irrespective of treatment,^[23] the response to chemotherapy is variable in different tumors. In response to chemotherapy some tumors regress completely, others show partial response and some even show frank resistance.^[24] Inability to predict the precise chemo-response before initiation of chemotherapy prompted us to evaluate the efficacy of a promising *in vitro* non-clonogenic assay, HDRA, to predict the response to chemotherapy.

HDRA, unlike the conventional monolayer culture, is a three-dimensional culture, performed on collagen gel matrix (a major matrix protein in the body), thereby better simulating the *in vivo* tumor milieu than spheroid cultures that are grown on agar. It simulates hypoxic tumor interior, its low pH and inaccessibility to chemotherapeutic agents. HDRA needs very small amount of tissue and can evaluate both growing and resting tumor cells, It has several advantages over the conventional clonogenic assays, such as higher positive predictive value (83% vs. 69%), better evaluability (83% vs. 30%), feasibility of multi-drug testing as well

as better quality; as the cell clumps can be distinguished from tissues.^[10,11] Tissue architecture and cell-to-cell contact are maintained when tumor specimens are incubated for seven days with chemotherapeutic agents and cell disintegration needed for plating clonogenic assays is not necessary in this assay. The concentration of individual drugs used and the duration of exposure *in vitro* were determined by the pharmacokinetics of the drugs. This technique has been used to study various biomarkers^[25] and to tailor chemotherapy for tumors of gut,^[6] breast,^[9] ovary^[7] and urological cancers.^[8] In a blinded study, the overall survival and disease-free survival of HDRA sensitive gastric cancer was found to be significantly higher than those of the HDRA-resistant ones.^[26] Similar results have been reported in head and neck squamous cancer.^[12]

In the present study, the evaluability of HDRA was fairly high at 91% but it is less than 100%, reported earlier in oral cancer^[14] and 98% reported in all head and neck cancers^[12] as five cases that showed infection were excluded from study. The strength of our study lies in the larger group of patients (52) and the fact that all tumors were subjected to the same set of drugs or their combinations. Using this technique, Ariyoshi *et al* reported an accuracy of 78.9% with 50% true negativity, 86.7% sensitivity, 50% specificity and 86.7% true positivity in oral cancers but 17/19 patients tested for individual drugs received combination chemotherapy and seven had radiotherapy also.^[14] Thirty out of 31 patients (97%) received one of the drugs or combination studied by HDRA, thereby making the interpretation more reliable. The accuracy of HDRA in predicting response to chemotherapy was similar to that reported earlier^[14] but our specificity and negative predictive values were better at 71% and 80%. In a relatively chemo-resistant tumor like oral cancer it is important to have a high negative predictive value (80%) that will spare a lot of patients, who are not likely to benefit from chemotherapy, from its unnecessary toxicity. Negative predictive value in the current study is better than those described earlier.^[11,14]

More recently chemosensitivity by collagen gel droplet drug sensitivity test (CD-DST) has been tried in oral cancer and was successful in 4/6 cases.^[27] In this simple and fast method, only a small number of cells are needed and easy quantification of the anticancer effects is possible without contamination with fibroblasts by using an image analysis system. Tono *et al*, evaluated the usefulness of *in vitro* assays systems for predicting the chemosensitivity of resected colorectal liver metastases and found chemosensitivity by collagen gel droplet drug sensitivity test (CD-DST) to be very low colorectal liver metastasis.^[28] By

using dissection under magnification it is possible to isolate and culture epithelial tumor cells and minimize fibroblast overgrowth even in HDRA.

To conclude, prediction of chemosensitivity by HDRA will help in optimizing the use of resources and in avoiding delays in initiating alternative treatment for non responders. At the same time the best drug or combination can be used for the responders. The results of this study will help us to develop a combined chemo-radio sensitivity model by incorporating *in vitro* radiation for patients undergoing chemo-radiation rather than surgery for organ preservation. This will have wider application in selecting patients and customizing regimen for their chemotherapy and concurrent chemo-radiation protocols as well as for identifying various biomarkers of chemo-radiation resistance. With sequential therapy gaining more popularity in the coming years in the management of head and neck cancer, HDRA will prove helpful in pre-treatment prediction of the anticipated response of individual tumors.

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