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ANALYSIS OF *IN VITRO* AND *IN VIVO* SENSITIVITY OF ORAL CANCER CELLS TO METHOTREXATE

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Aim: **The present study was directed on the assessment of the response of treatment-naive oral cancer cells to methotrexate (MTX) *in vitro* and clinical response to MTX therapy.** ***Methods:*** **A pilot study of *in vitro* evaluation of MTX response of oral cancer cells from 10 patients was conducted using a cell viability assay to determine the sensitivity/resistance to MTX. Quantitative *in vitro* data were correlated to the clinical outcome to MTX therapy.** ***Results:*** **A positive correlation was observed between the effect of MTX on tumor cells *in vitro* and clinical response for 7 out of 10 patients.** ***Conclusions:*** **Observations from the proof-of-principle pilot study suggests that oral cancer cells have intrinsically variable response to MTX. Confirmation of these findings with a larger cohort of patients could aid in the development of individualized therapies for this class of malignancy.**

Key Words: **oral cancer, methotrexate, intrinsic, drug sensitivity/resistance.**

Resistance to anticancer drugs used in the clinic has led to the persistence of tumor growth and the failure of chemotherapeutic regimens. Methotrexate (MTX) has been used as a chemotherapeutic agent for the management of tumors of varied origins. The patients presenting with oral carcinoma at our institute are predominantly habitual chewers of pan, areca nut and tobacco [1, 2]. Characterization of this class of neoplasia with unique etiology showed that the majority of these neoplasia are squamous cell carcinoma (SCC) and they express certain tumor-specific markers such as the carcinoembryonic antigen [3]. For oral cancers patients (prevalently of T3/T4 stages), MTX is administered to reduce the tumor mass for further management with radiotherapy and/or surgery and as a palliative option. Earlier studies on administration of MTX as a single agent or in combination with other chemotherapeutic agents as well as other modalities of therapy such as radiation has shown varying degrees of success for head and neck cancers [4–6].

A number of factors are critical for a favorable clinical outcome of MTX therapy. These include elevated levels of the target, dihydrofolate reductase (DHFR), diminished polyglutamation and transport of MTX as well as altered binding affinity of DHFR to MTX. Translational control of DHFR, including feed back mechanisms in DHFR biosynthesis could also contribute to MTX resistance [7, 8]. Multiple mechanisms for MTX resistance in a clinical setting for head and neck carcinoma has been proposed [9], and the implications of the various pathways for clinical MTX resistance has also been reviewed [10].

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Abbreviations used: 5-FU – fluorouracil; DHFR – dihydrofolate reductase; HBSS – Hanks balanced salt solution; MTX – methotrexate; SCC – squamous cell carcinoma; WBC – white blood cells.

The current study was undertaken to evaluate the sensitivity of oral cancer cells to MTX *in vitro* and its association with clinical response to MTX in oral cancer patients.

Patient selection and treatment. Patients were treated at the Dental Division of Kidwai Memorial Institute of Oncology, Bangalore, India and were randomly included in the study. Informed patient consents were obtained, and approved treatment protocols were administered. In addition, patients who had advanced disease were put through a screening committee to start chemotherapy for palliation. All the patients (35 to 65 years old) recruited in the study received chemotherapy with weekly MTX injections at the dose of 50 mg IM. A maximum of five injections were administered. During the treatment period, the patients were monitored for total WBC count, nausea, vomiting and mucositis. All clinical assessments of the response were made after the chemotherapy phase to evaluate the correlation between the *in vitro* sensitivity to MTX and tumor response. Some patients subsequently received radiotherapy, two patients received radiotherapy prior to MTX therapy.

Criteria for clinical response. The following criteria were used for the assessment of clinical response. Complete response: total disappearance of the clinically viable lesion; partial response: > 50% reduction in tumor size; minimal response: < 50% reduction in tumor size; and no response: stable disease.

Chemicals used in the assay. Trypan blue, Collagenase IV, Hanks Balanced Salt Solution (HBSS) were all obtained from Sigma Chemical Co (St. Louis, MO, USA). MTX was a kind gift from Dr. R.M. Lalitha. MTX dissolved in L-15 tissue culture media was used in the study. The L-15 tissue culture medium with composition identical to that of GIBCO BRL, USA was used. Final assay medium contained L-15 medium supplemented with 10% fetal bovine serum (FBS).

Preparation of cell suspensions from tumor tissue. Tumor biopsy samples prior to treatment were

placed in HBSS at 4 °C. To generate tumor cell suspensions from the biopsies, the tissue was minced using scissors in a watch glass. The minced tissue was suspended in 0.075% collagenase for 30 min at 37 °C to obtain single cell suspensions for the assay. The larger clumps were discarded after the initial centrifugation (27 x g). The supernatants were further subjected to a centrifugation at 475 x g. An aliquot of the cell suspension was mixed with equal volume of trypan blue solution, mixed gently and placed in a hemacytometer, and the number of viable cells in the suspension.

Drug sensitivity assay. Cultured oral cancer cells were maintained in L-15 media supplemented with 10% FBS, and treated at three different concentrations of MTX (0.25 μM, 25 μM, and 75 μM) for 24 h at 37 °C. The highest concentration was selected based on the use of similar concentrations for MTX-resistant human cells in earlier studies [11].

Assessment of cell viability in vitro. After 24 h of incubation with MTX, aliquots of cell samples were mixed gently with equal volume of trypan blue dye solution and immediately examined using light microscopy. Viable cells were counted in triplicates for each MTX concentration per sample. Results were presented as mean ± standard deviation.

Criteria for in vitro sensitivity. The cells were scored as sensitive, moderately sensitive, moderately resistant and resistant, based on the LC_{50} values (LC_{50}: lethal concentration inducing 50% of cell death). If the LC_{50} was achieved at the lowest concentration (0.25 μM), the tumor cell populations were considered as sensitive, whereas inability to attain LC_{50} even at the highest concentration used (75 μM) would render them to be classified as resistant. If LC_{50} was in the proximity of 25 μM MTX the tumor cells were designated as moderately sensitive. Attaining LC_{50} at the highest concentration (75 μM) rendered them to be classified as moderately resistant.

The study was performed on 10 oral tumors of T4 stage (50%), T3 stage (30%), or T2 stage (20%); staging from T2N0 to T4N3 (Table 1). Four patients presented with carcinomas of the buccal mucosa and four with carcinoma of the alveolus. There was one presentation with carcinoma of the floor of the mouth, and one locoregional extension of the carcinoma of the buccal mucosa to tongue.

Table 1. Clinical characteristics of patient samples used in the study

Patient No.	Age	Sex	Site of Lesion#	Stage of Tumor	Histological type, grade
1	45	F	Ca BM	T4N3	Squamous carcinoma
2	52	M	Ca tongue (L)	T2N0	Verrucous
3	42	F	Ca BM (R)	T3N1	Squamous carcinoma, Gr II
4	35	F	Ca alveolus(L)	T4N1	Squamous carcinoma, Gr II
5	45	F	Ca alveolus(L)	T4N1	Squamous carcinoma, Gr III
6	55	F	Ca alveolus(L)	T3N1	Squamous carcinoma, Gr III/IV
7	58	F	Ca alveolus(L)	T4N0	Squamous carcinoma, Gr II
8	65	F	Ca BM (R)	T3N1	Verrucous
9	60	M	Ca floor of mouth	T4N1	Squamous carcinoma, Gr III
10	35	F	Ca BM (R)	T2N1	Squamous carcinoma, Gr II

Notes: #BM – buccal mucosa; R – right; L – left.

Differential sensitivity to MTX was observed among the various tumor cells in the *in vitro* assay, and these data were compared to the clinical outcome (Figure and Table 2). In case 1, tumors cells were found to be sensitive to MTX, and the laboratory data from patient 1 corresponded to a good clinical response. In case 2, *in vitro* response of tumor cells was moderately sensitive, but clinical response in that patient was good. In cases 3 and 7, moderate resistance of tumor cells *in vitro* and moderate response or good response in clinical conditions were registered, respectively. Tumor cells from cases 5, 6, 8–10 were resistant to MTX *in vitro*, and in all these cases clinical response on MTX therapy was moderate or absent.

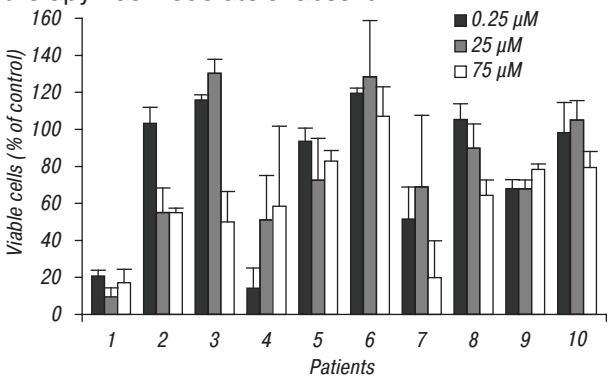

Figure. The cell viability was assessed by using trypan blue dye exclusion assay. Mean of triplicate treatments ± standard deviation is represented. Assays using tumor cells without inclusion of MTX served as controls. Viable cells from controls were considered as 100% to determine the effect of MTX

Table 2. Correlation between *in vitro* sensitivity and clinical response to methotrexate

Patient No.	Treatment	Clinical response to Methotrexate treatment	Laboratory analysis (LC_{50}, μM)
1	CT	Good response	Sensitive (< 0.25)
2	CT	Good response	Moderate Sensitive (~25)
3	CT	Moderate response	Moderate resistance (~75)
4	CT	50% response	Variable (NE)
5	CT	Moderate response	Resistant (> 75)
6	CT	Resistant	Resistant (> 75)
7	CT	Good response	Moderate resistance (< 75)
8	CT	Resistant	Resistant (> 75)
9	RT + CT	Resistant	Resistant (> 75)
10	RT + CT	Resistant	Resistant (> 75)

Note. NE – not estimated.

Failure of anticancer chemotherapy is largely attributed to intrinsic or acquired resistance to anticancer drugs. Our studies using cells from biopsies of oral tumors from patients prior to initiation of MTX treatment depict existence of intrinsic resistance to the drug in a number of cases.

Our observations with oral carcinoma cells suggest, for the first time, that the intrinsic resistance to MTX could be a contributing factor in the lack of response of oral carcinoma to MTX in the clinic. The implications of various chemosensitivity assays and their extrapolation to *in vivo* scenario have been discussed in detail elsewhere [12, 13]. Studies on drug sensitivity of advanced cancers of gingivo-buccal, tongue and floor of the mouth to various drugs showed that 52% of the tumors tested showed sensitivity to MTX with a good clinical correlation [14].

Future studies on a larger patient population could provide valuable information with respect to the generality of intrinsic MTX resistance in oral cancers in South India. Further, *in vitro–in vivo* correlation from such a study could provide directions for designing individualized therapies for this unique class of neoplasia.

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