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Research Article

MULTI-NUCLEATION INDICES IN RADIOTHERAPY TREATED SQUAMOUS CELL CARCINOMA- A TOOL IN TREATMENT PLANNING AND ASSESSMENT OF PROGNOSIS

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A B S T R A C T

Background: Oral cancer ranks as one of the top ten cancers worldwide being the most prevalent cancer in India. Radiotherapy, one of the modality of treatment causes damage to the DNA and target cells through complicated series of atomic interactions. The present study was undertaken with an aim to evaluate the multi-nucleation in malignant cells obtained from the scrapings taken from the site of lesion.

Materials and method: Fifty patients attending outpatient department of JIPMER hospital with histopathologically confirmed Squamous Cell Carcinoma (SCC) of oral mucosa and undergoing radiotherapy alone were included. From all these patients the specimens from the site of lesion was collected on pre-treatment day and subsequently on 2nd, 7th, 12th and 30th day of radiotherapy.

Results: A rise of 45.18% was observed at 4 Gy with peak at 14 Gy as 89.61%, a sudden fall to 27.11% at 24 Gy and a fall of 133.15% in multi-nucleation were observed at 60 Gy. The mean percentage increase when compared with pre-treatment day was statistically significant (p=0.001) for comparison between day 0 (pre-treatment) & day 2, similarly 0 & 7, 0 & 12 and finally 0 & 30.

Conclusion: The progressive increase in Multi-nucleation indices with increasing dose of radiation indicates that this parameter can be used as an indicator for assessing the response of tumour of radiotherapy.

INTRODUCTION

Oral cancer ranks as one of the top ten cancers worldwide. In India it is the third most common cancer among females and frequent in males making it one of the most prevalent cancer type[1]. Among oral cancers, Squamous Cell Carcinoma (SCC) is the most common histological type. It is observed to develop after the age of 50 having a peak in the sixth decade of life[2]. The treatment modalities for oral SCC include management with surgery, radiotherapy, along with chemotherapy or various combinations of these modalities are administered depending on the presentation, pathological findings and grading [3]. The estimation of radiosensitivity of individual tumours is essential not only to opt for the treatment, but also for planning the optimum radiation schedule for each patient. But the dilemma is that radio-sensitivity is not uniform; even in the similar histological subgroups in head and neck cancers. The in-vivo cytological tests and fraction of the cell surviving after 2 Gy dose of radiation are established means for forecasting radiation response[4]. Alternate methods are used for serial cytological evaluation of nuclear abnormalities in carcinoma patients.[5] Response of malignant cells to radiation therapy was assessed by various cytological changes in the nucleus such as nuclear enlargement, micro nucleation, nuclear budding, multi-nucleation, bi-nucleation, karyorrhexis, karyolysis, vacuolization and granulation. Radiation induced changes were first reported by Ameson *et al* in the year 1935.[6] The irradiation effects on mucosal cells of oral cancer patients were identified and gradually the abnormal forms of nucleus observed were named as Pyknosis, Nuclear budding, Multi-nucleation (1960).[7,8] The recent studies have also tried to find the molecular origin of these alterations [9]. The escalating increase in Micronucleus, Multi-nucleation, Karyorrhexis and Karyolysis indices with increasing dose of radiation proves that these parameters can be used as indicators for assessing the response of tumour after radiotherapy. The present study was undertaken to establish the relationship between Multi-nucleation indices with radiation dose and to investigate the prospect of utilizing them as an assay to predict tumour response to radiotherapy in oral cancers.

MATERIALS AND METHOD

The study was conducted on the patients referred for treatment from surgery and E.N.T. OPD for radiotherapy, from the outpatient department of JIPMER hospital. The clearance institutional ethical committee was received beforehand. Fifty patients (age range of 30-65 yrs) with histopathologically confirmed Squamous Cell Carcinoma of oral mucosa were included in the present study those who were treated by radical radiotherapy alone. Each patient received 4, 14, 24 and 60 Gy at 2nd, 7th, 12th and 30th day respectively. Any patient treated with other modalities, like surgery or/and chemotherapy, along with radiotherapy or having radiation schedules different from the above mentioned were excluded from the study.

A standard Performa was prepared in order to record the history and general physical examination in respect of each case. From all these cases the specimens from the site of lesion (buccal mucosa, alveolus, retro molar area) was collected and slides were prepared following the protocol given by Halder *et al* [10] for the diagnosis and confirmation of carcinoma. It was carefully ensured that scraping was only taken from the tumour site, avoiding adjacent normal mucosa. Pre-treatment scrape smears were collected from the site of lesion in each patient. Subsequently 3 to 4 smears were prepared from material collected from each patient after delivery of various radiotherapy fractions, i.e. after the 2nd, 7th, 12th and 30th day.

Specimen collection

The included patients were asked to rinse their mouth scrupulously and following that the material was collected from the oral cavity by scraping the buccal mucosa on the affected with a previously wetted wooden spatula and the material was immediately spread on clean glass slides and smeared. After air drying slides were placed in freshly prepared fixative in the proportion of 3 parts of methanol and one part of glacial acetic acid for 20 minutes. These fixed slides were stained with May-Grunwald and Giemsa stain.

Staining Procedure

These slides were air dried and fixed with methanol and the stained with Glemsa and May-Grunwald's stain. The fixed slides were kept in May-Grunewald stained for 5-7 minutes. The slides were then washed and counter-stained with Giemsa stain for 8-10 minutes, followed by washing with distilled water and the stained slides were mounted with cover slip and left undisturbed overnight. The slides were observed for nuclear abnormalities under bright field Nikon microscope under various magnifications.

Observations were recorded and tabulated. Photomicrographs showing various nuclear anomalies were taken. Around 500-1000 cell were evaluated from each sample collected on each fraction and the results is expressed in terms of 500 tumour cells with normal nucleated cells.

Multi-nucleation (MNU) was defined as more than two nuclei in a single cell with no micronucleus or nuclear budding (Fig 1c). For other nuclear changes, the criteria used for identification were predefined with Micronucleus (MN) being intra-cytoplasmic, DNA staining bodies having slightly lesser staining intensity, less than one-third the size the main nucleus and in vicinity of nucleus but distinctly separate from it (Fig 1a) and Nuclear budding (NB) being bodies similar to micronuclei except for the fact that their separation from the main nucleus was indistinct (Fig 1b).

Analysis Procedure

Five hundred cells from the prepared smears of each patient were assessed for various radiations induced nuclear changes at 4, 14, 24 and 60 Gy and were compared. Variance was analyzed within the group and p-value was calculated. This was analyzed by Kruskal-Wallis One-way (Anova-test).

Observations and Results

Out of the 50 cases included in the present study 37 (74%) were males and 13 (26%) were females (Ratio 3:1). The majority number of patients were in age group of 51-60 yrs (54%) followed by 20% in 41-50 years, least 10% were in age group of 31-40 years (Table 01).

Table No.01 Case Distribution With Age & Sex

Age Range (yrs)	Males	Females	Total
31-40	04	01	05
41-50	07	03	10
51-60	20	07	27
>=61	06	02	08
Total	37	13	50

There is marked increased noted in the mean values of MNU at 4 Gy. At 14 Gy radiations, an increase of 30% in MNU which further rose to 62% at 14 Gy (Table 02). MNU showed a further hike of 18% at 24 Gy with a gross fall of 90% was observed at 60 Gy in MNU.

Table No. 2 Showing % Rise In Multinucleation Indices

Day Of Rt	Rt Dose (GY)	MNU count /500 cells	
		Mean± sd	Degree rise %
0	0	34.08±3.999	--
2	4	49.48±4.704	45.18
7	14	80.02±5.587	89.61
12	24	89.26±4.549	27.11
30	60	43.88±6.286	-133.15

Relative increment % was calculated for the nuclear abnormality

$$\text{Nuclear anomaly \%} = \frac{\text{No. of cells after radiation}}{\text{No. of cells prior to treatment}} \times 100$$

Table No. 3 showing the p-value at various comparison levels with various dosage of Radiotheraphy with pretreatment Multinucleation indices

DAYS	RT DOSE (Gy)	MNU	
		MEAN±SD	t-TEST
0 & 2	2	-5.400±6.652	.000
0 & 7	14	-5.940±9.927	.000
0 & 12	24	-5.180±7.024	.000
0 & 30	60	-9.800±5.147	.000

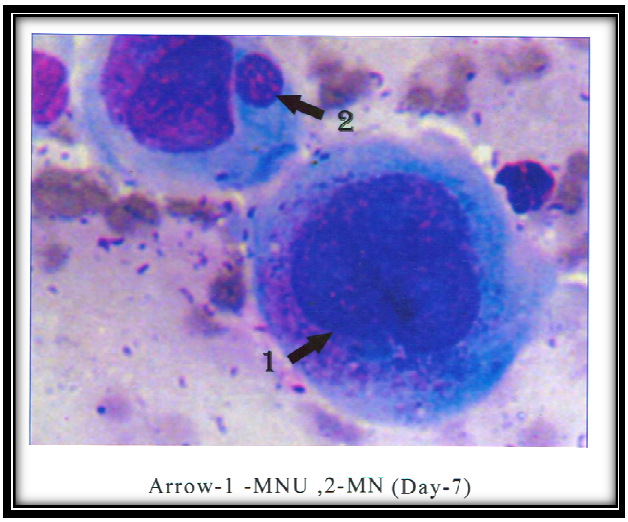
Table No. 4 Relative Increment (RI) For Multinucleation Indices

DAYS	RT DOSE	MEAN MNU	R.I.
0	0	34.08	-
2	4	49.48	145
7	14	39.64	116
12	24	52.40	154
30	60	65.00	2

A marked increase is noted in relative increment index from 145 MNU at 4 Gy with escalation upto 154 at 24 Gy and a fall to the relative increment index falls down to a level to 2 at 60 Gy (Table 04).

Multi-nucleation

A rise of 45.18% was observed at 4 Gy as mentioned in Table no. 01. A peak in the multi-nucleation was observed at 14 Gy as 89.61% then with a sudden fall to 27.11% at 24 Gy and a fall of 133.15% in multi-nucleation was observed at 60 Gy. The mean percentage increase when compared with pre-treatment day was statistically significant (p=0.001) for comparison between day 0 (pre-treatment) & day 2, similarly 0 & 7, 0 & 12 and finally 0 & 30.



Colour Plate 01 Cells showing Multinucleation (arrow 1) and Micronucleus (arrow 2) formation after 14 Gy radiation.

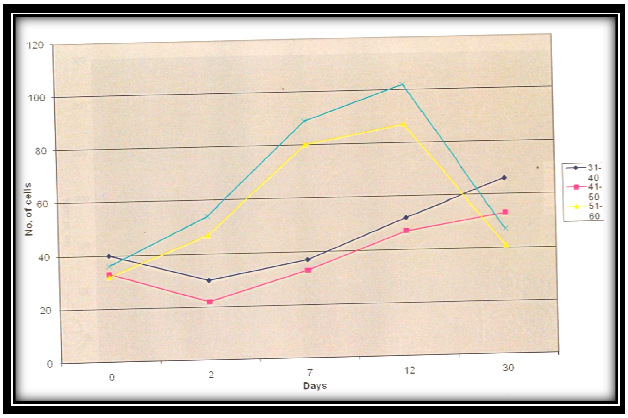


Figure 1 Multinucleation at 4, 14, 24 and 60 Gy respectively at 2nd, 7th, 12th and 30th daygiven for different age groups (31-40 yrs, 41-50 yrs and 51-60 yrs and an average given as blue line).

DISCUSSION

Radiotherapy is the use of ionizing radiations to treat malignant lesions by causing damage to the DNA and target cells through complicated series of atomic interactions. Most of the nuclear damage is due to the generation of free radicals by the interaction of the radiation with water molecules which in turn interacts with and damages the DNA.

In the past, serial cytology has been done in order to find a standard method for the prediction of response to radiotherapy or prognosis of oral cancers by recording the radiation induced cell damage.[1] Response of malignant cells to radiation therapy was assessed by various cytological changes in the nucleus or cytoplasm, named as nuclear enlargement, nuclear budding, micro nucleation, bi-nucleation multi-nucleation, karyorrhexis and karyolysis.

The present study was undertaken with an aim to evaluate the changes in malignant cells obtained from the scrapings taken from the site of lesion and not from the surrounding tissues. In many previous studies [11, 12] the changes were evaluated in benign cells collected from buccal mucosa around the tumour, whereas in this study it was assessed by the induction of various nuclear abnormalities in oral carcinoma patients by taking the smears from the site of lesion as we were looking for nuclear changes in the malignant cells.

A total of 50 histopathologically confirmed cases of SCC were included in the present study. 54% of the total cases are in 51-60 years age range and the incidence of oral carcinoma was more in males than in females, 74% and 26% respectively. As indicated in the table no. 01. Our findings are in accordance with recent literature findings which state that age-standardised incidence rates when stratified by sex were lower in females than males [13], and the male to female ratio is also showing a slow decline, as there is rising incidence in oral cancers in women.[14, 15] In our study, we found the ratio to be 3:1 which is nearing the ratio cited in literature.[16]

Serial cytology have been conducted earlier in order to find a standard method for the prediction of response to radiotherapy or prognosis of oral cancers by recording the radiation induced cell damage.[1] The commonly observed changes are multi-nucleation and nuclear enlargement of the malignant cells. Initially, Silverman *et al*. stated that multi-nucleation is a very frequent change in oral cancers brought about by radiotherapy; this was later endorsed by other researchers. [17,18]

Radiation induced multi-nucleation has been noted in cell cultures and animals experiments as well. Mehrotra *et al* observed that the frequency of multi-nucleation was increased with increased radiotherapy dosage in a serial scrape smears from both in normal mucosa and malignant sites. They also reported a significant association between multi-nucleation present in normal mucosa and that collected from malignant site and radiation dose ($p < 0.001$).[19] Similarly, it has been variously reported that the frequency of multinucleated cells in tumoral area in irradiated serial smears was increased with increased radiotherapy dosages[20,21]. Past radiobiological studies have also shown that not only is the induction of cell multi-nucleation dose dependent it also correlated with cell survival assay suggesting the change to be non-clonogenic.[22] In our study multinucleated cell showed a maximum rise from 45.18% at 4 Gy to 89.11% at 14 Gy thereafter the rise was only 27.11% and a gross fall of 133.15% was observed at 60 Gy (Table no.02). Two mechanisms responsible for radiation induced multi-nucleation have been proposed, including radiation induced per-oxidation of membrane lipids causing failure of cytoplasmic division leading to formation of a bi-nucleated cell which on further cell division would lead to multinucleation.[17] Another proposed mechanism suggests that due to radiation induced damage to pericentriolar matrix there is multipolar mitosis.[19] In our study the maximum increase in the multinucleated cells at 14 Gy would probably be the result of these mechanisms but a fall at 60 Gy could be explained on the basis that this massive damage leading to karyorrhexis. Many studies have reported that irradiated cells lose their proliferative property which might be because of the fact that

hardly any DNA is left after 4 weeks of treatment for cell division.[23]

When the paired t test was applied to the mean values of MNU at various dosage of radiotherapy significant p value ($p<0.0001$) in all the indices was obtained between pre treatment and after radiation therapy as indicated in table no.03. This finding is in agreement with the findings of the previous authors.[21] A very similar study was done by L. Bindu *et al* in which as many as 15 parameters were evaluated, out of which 7 parameters KR, pyknosis, KL, cytolysis, micro nucleation, nuclear buds and multi nucleation showed statistically significant results. [2] But the evaluation groups did not include 30th day of RT which in present study is included to evaluate the degree of maintenance of effect of RT.

Previous multiple studies with multi-nucleation and its assays during the first few days of radiotherapy have also shown that serial cytology has significant correlations with radiosensitivity. [24, 25]

Another finding which is apparent in the present study is a nonlinear increase in the multi-nucleation indices on exposure to increasing doses of radiotherapy. It can be observed from the graphs (Figure 1) similar findings have been shown in a study by V Raj *et al* [26].

Multi-nucleation indices is a useful tool in the assessment of biological damage that can help in assessing the radiosensitivity of tumour since previous studies point on the fact that the tumours which were radioresistant exhibited lesser degree of change as compared to radiosensitive tumours. The damage to nuclear membrane has been suggested as a mechanism that directs to cell death and as a result multinucleated cells are considered to be dead cells and unable of giving rise to colonies. [27]

Relative increment percentage was calculated for all the nuclear parameters taken in the study as per the formula given along with the table no.06. The obtained values shows a marked increase with each dosage of radiotherapy in MNU till 24 Gy and at 60 Gy a marked fall was observed and it remained only two in MNU which was 154 at 24 Gy. The Multi-nucleation index varies with age and in age group 31-40 yrs, 41-50 yrs the observed data shows an increment at 12 and 30 days but the older age group has a drop after 12 days (figure 1).

CONCLUSION

It is ironic that despite the advancements in cancer treatment there is only a little change in the mortality from epithelial or Squamous Cell Carcinoma (SCC) of oral cavity. The present study was aimed to explore the possibility of establishing a relationship between the frequencies of Multi-nucleation indices in patients with oral cancer with applied dosage and duration of radiotherapy.

The progressive increase in Multi-nucleation indices with increasing dose of radiation indicates that this parameter can be used as an indicator for assessing the response of tumour of radiotherapy. The measurement of relative increment index shows a sustained increase with increasing dosage of radiation. In order to differentiate between the radio-resistant and radiosensitive tumors, the Multi-nucleation indices taken at 4 Gy may be used to select the line of treatment. This parameter can be used as prognostic indicator in oral carcinoma cases undergoing radiotherapy. The level of response of tumour to radiotherapy as assessed on 7th day can be used for bringing out alteration or modifications in the further treatment.Since the study was done in a limited number of cases due to time constraint so the translation for the general population is not mentioned but certainly based on the results we may do so and take the study of a greater population for its authentication. As nuclear changes are observed even at 4Gy and they peak by the time 24Gy dose is delivered, thereafter nuclear changes are decreased. So the MNU can be used as a predictor for assessing radio sensitivity and needs further study.

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