



Evaluation of Radiation-induced Cytological Changes in Lesional Oral Cancer Cells and Adjacent Normal Mucosal Cells

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ABSTRACT

Aim: To assess various cytological changes for predicting radiosensitivity of oral squamous cell carcinoma by exfoliative cytology.

Materials and methods: Histologically proven 30 cases of oral squamous cell carcinoma who underwent fractionated radiotherapy in a dose of 45-60 Gy in 5 fractions/week were enrolled in the study. The exfoliative cytology smear was evaluated on lesional and adjacent oral mucosa before radiotherapy, during radiotherapy (8 and 11th fraction) and post radiotherapy (4, 6 and 8 weeks). Various parameters like multinucleation, cellular enlargement, nuclear enlargement, cytoplasmic vacuolation, cytoplasmic granulation, leukocytic infiltration were evaluated.

Results: Statistical significant values were seen in the inter-group comparison of all the parameters when compared adjacent mucosa and normal mucosa for leukocytic infiltration in pretreatment smear.

Conclusion: The study showed that radiation-induced cytological changes in oral squamous cell carcinoma have a significant dose-related increase. This dose-response relationship and the high intratumoral variations suggest that serial assay of these changes has potential use for radiosensitivity prediction.

Clinical significance: Radiosensitivity prediction can be evaluated by means of cytological smears in one stop crisis centre (OSCC) individuals subjected to fractionated radiotherapy by evaluating the cytological parameters.

Keywords: Cytology, Oral cancer, Radiosensitivity.

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INTRODUCTION

Exfoliative cytology is the microscopic examination of shed cells from an epithelial surface.¹ It has been known for more than 100 years that cells removed from tumors can provide a clue to diagnosis,^{2,3} but it was not until Papanicolaou,⁴ and Traut⁵ and others^{6,7} who explored the diagnostic potential of the fact that, exfoliative cytology can be extensively applied to the detection of cancer.⁸

Oral cancer is a serious and growing problem in many parts of the globe. More than 90% of oral malignancies in the upper aerodigestive tract are squamous cell carcinomas.⁹

As a standard treatment for oral squamous cell carcinoma radiotherapy is frequently used which can either be alone or in combination with surgery.¹⁰ Evaluation of radiation-induced cellular changes with a view to predict radiosensitivity has led to interest many investigators as such changes were first documented in biopsied material in 1935.¹¹ A cytologic test (the radiation response test) for this in "non-malignant appearing cells" from vaginal smears was developed in 1947.¹² Both success¹³ and failure¹⁴ were later found by others in cancer of cervix.

Evaluation of cytologic changes due to irradiation on oral mucosa was first described in 1957 and on oral cancer in 1959.¹⁵ Later, however, reports indicated no paucity of correlation of the changes either with dose or tumor radiosensitivity.^{16,17}

By 1960s pyknosis, karyorrhexis, karyolysis, enlargement, multinucleation, and crenation of nuclear membrane were the nuclear morphological changes that were evaluated by

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cytology and later became well established.¹⁸ Cytologically, malignant as well as benign cells show identical changes, except for cancer cells depict significant hyperchromasia with the increased relative nuclear area, coarse irregularly distributed chromatin and irregular nuclear outlines.^{19,20} Whereas, cytoplasmic vacuolation, multinucleation, bizarre cell formation, leukocytic infiltration, nuclear enlargement with clumping of chromatin and wrinkling of the nucleus are few of the other cellular changes which have been described to occur following post radiotherapy.²¹

The estimation of radiosensitivity of individual tumors will be essential for planning the optimum radiation schedule for each patient and in choosing the treatment.²² Evaluating the cytological changes for the sensitivity of oral squamous cell carcinoma in patients undergoing radiotherapy will help us observe the cytological changes before, during and after radiation. It will also help predict the firmness of relationship amongst dose and duration of radiation therapy of these changes.

This study was undertaken to determine if serial cytological evaluation done before during and post-radiotherapy, in oral squamous cell carcinoma patients can predict radiosensitivity or not.

MATERIALS AND METHODS

A total of thirty patients with histologically proven cases of oral squamous cell carcinoma who had reported to Department of Radiotherapy and Oncology and underwent fractionated radiotherapy in a dose of 45 to 60 Gy in 5 fractions/week (total 30 fractions), were enrolled in the study.

Inclusion Criteria

- Patients undergoing fractionated radiotherapy in a dose of 45 to 60 Gy in 5 fractions/week. (Total 30 fractions).
- Patients undergoing the only radiotherapy for oral squamous cell carcinoma and not been treated with other modalities, like chemotherapy/surgery, along with radiotherapy during the course of study.

Exclusion Criteria

- Patients having different radiation schedules from the above mentioned.
- Patients who fail to turn up for follow-up on the intervals of the study.

The OSCC patients were evaluated in the following schedule:

- Pretreatment Smear
- During radiotherapy 8th fraction
- During radiotherapy 11th fraction
- Four weeks post-radiotherapy

- Six weeks post-radiotherapy
- Eight weeks post-radiotherapy

At each visit, the patients were asked to rinse their mouth scrupulously and following that the material was collected from the oral cavity by scarping the lesional site and the adjacent mucosa with a sterile wooden spatula which was moistened in distilled water to avoid discrepancies. Normal appearing mucosa just adjacent to the lesional site was considered in the study. For the preparation of smear during every visit two new sterile wooden spatulas were used, one for the lesional site and other for adjacent mucosa. The study included analysis of total six smears from OSCC lesional site and six smears from adjacent mucosa. The obtained materials were directly smeared on clean glass slides (different slides were used for lesional and adjacent mucosa). Further, immediately fixed in cytofixative solution, while they were wet, and sent to the laboratory for staining. These fixed slides were stained with Papanicolaou stain.

Analysis of the Smears

A total of 500 cells were evaluated by a single observer from the samples collected on each occasion. Smears were examined at both 20x and 40x with an eyepiece of 10x of a light microscope. The nuclear and cytoplasmic changes observed were: Nuclear enlargement, cellular enlargement, multinucleation, cytoplasmic vacuolation, cytoplasmic granulation, and leukocytic infiltration. Cell clumps, cells with indistinct nuclear membranes and poorly stained cells were not counted.

RESULTS

Statistical Analysis

Data was gathered, categorized and coded. Data were analyzed using SPSS-16 software using Independent t-test and repeated measures analysis of variance (ANOVA) test.

Graph 1 reveals the frequency distribution of gender among study. Out of 30 individuals enrolled there were 20 male and 10 female patients.

Table 1 reveals descriptive statistics for age in the study group with a mean age of 57.37 years.

In the present study, the cytological evaluation of the lesional and the adjacent mucosal site collected at various intervals was considered in the groups.

Thus, two major groups based on smear collection are:

- Lesional Mucosa
- Adjacent Mucosa

Tables 2 to 6 reveals intergroup comparison of multinucleation, nuclear enlargement, cellular enlargement, cytoplasmic vacuolation, cytoplasmic granulation scores

among lesional mucosa and adjacent mucosa by Independent t-test. Amongst each group (adjacent mucosa group and lesional group) intragroup comparison for cellular

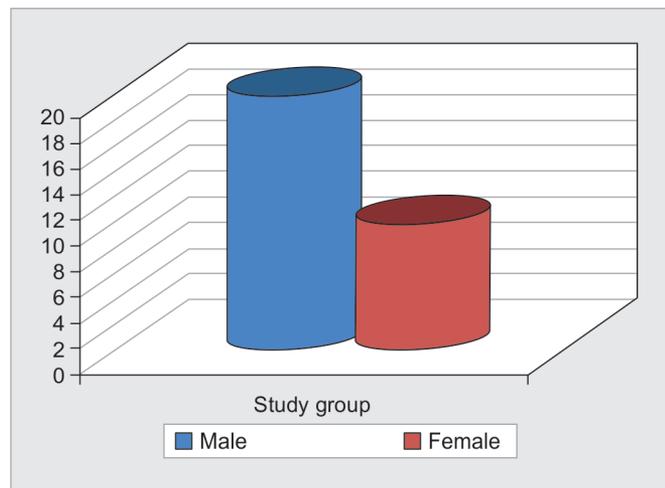
arrangement at a different time interval (before, during, post-treatment) was done by repeated measures ANOVA. There is evidence of statistical significance among all the groups.

Table 7 reveals intergroup comparison of leukocytic infiltration scores among lesional mucosa and adjacent mucosa by Independent t-test. Statistical significance is evident in during treatment and posts radiotherapy group. No evidence of statistical significance in adjacent mucosa and lesional mucosa in pre-treatment smear.

DISCUSSION

India has one of the highest incidence of oral cancer making it the most common cancer among men (men: women ratio 2:1) and accounts for about 30% of all new cases annually.²³ A recent survey of cancer mortality in India shows cancer of the oral cavity as the leading cause of mortality in men and responsible for 22.9% of cancer-related deaths.²⁴

The overall 5-year survival rate for all stages of oral cancer is 60%. These rates are better for localized tumors as compared to tumors with regional or distant metastasis.²⁵



Graph 1: Frequency distribution of gender among study

Table 1: Descriptive statistics for Age in the study group

Descriptive statistics					
	N	Minimum	Maximum	Mean	Std. deviation
Age	30	32	90	57.37	12.489

Table 2: Intergroup comparison of multinucleation score among lesional mucosa and adjacent mucosa by Independent t-test

	Period of study			p-value repeated measures ANOVA
	Pretreatment	During treatment	Post radiotherapy	
Adjacent	0.07 ± .254	1.1667 ± .83391	3.5333 ± 1.25212	<0.001
Lesional	4.33 ± 1.918	14.2000 ± 3.89872	39.4000 ± 9.69749	<0.001
Mean difference	-4.267	-13.03333	-35.8667	
t-value	-12.080	-17.905	-20.091	
p-value	<0.001	<0.001	<0.001	

Intra group comparison for multinucleation at different time intervals (pre vs during vs post) were done by repeated measures ANOVA

Table 3: Intergroup comparison of nuclear enlargement score among lesional mucosa and adjacent mucosa by Independent t-test

	Period of study			p-value repeated measures ANOVA
	Pretreatment	During treatment	Post radiotherapy	
Adjacent	1.83 ± 1.510	11.8000 ± 4.74451	14.3667 ± 6.89569	<0.001
Lesional	26.93 ± 4.884	66.6667 ± 9.78505	97.5000 ± 16.89675	<0.001
Mean difference	-25.100	-54.86667	-83.13333	
t-value	-26.890	-27.635	-24.951	
p-value	<0.001	<0.001	<0.001	

Intra group comparison for nuclear enlargement at different time intervals (pre vs during vs post) were done by repeated measures ANOVA

Table 4: Intergroup comparison of cellular enlargement score among lesional mucosa and adjacent mucosa by Independent t-test

	Period of study			p-value repeated measures ANOVA
	Pre-treatment	During treatment	Post radiotherapy	
Adjacent	2.13 ± 1.432	10.8667 ± 4.28899	14.4667 ± 6.17968	<0.001
Lesional	23.43 ± 4.224	59.7000 ± 7.20704	83.2333 ± 7.62339	<0.001
Mean difference	-21.300	-48.83333	-68.76667	
t-value	-26.159	-31.892	-38.381	
p-value	<0.001	<0.001	<0.001	

Intra group comparison for cellular enlargement at different time intervals (pre vs during vs post) were done by repeated measures ANOVA

Table 5: Intergroup comparison of cytoplasmic vacuolation score among lesional mucosa and adjacent mucosa by Independent t-test

	Period of study			<i>p</i> -value repeated measures ANOVA
	Pretreatment	During treatment	Post radiotherapy	
Adjacent	0.13 ± 0.507	1.5000 ± 2.93316	2.2000 ± 3.69902	<0.001
Lesional	2.40 ± 0.894	11.6667 ± 1.60459	39.8667 ± 2.56949	<0.001
Mean difference	-2.267	-10.15667	-37.6667	
t-value	-2.073	-16.655	-45.807	
p-value	< 0.001	< 0.001	< 0.001	

Intra group comparison for cytoplasmic vacuolation at different time intervals (pre vs during vs post) were done by repeated measures ANOVA

Table 6: Intergroup comparison of cytoplasmic granulation among lesional mucosa and adjacent mucosa by Independent t-test

	Period of study			<i>p</i> -value repeated measures ANOVA
	Pretreatment	During treatment	Post radiotherapy	
Adjacent	2.20 ± 1.095	10.1000 ± 1.47040	2.2000 ± 3.69902	<0.001
Lesional	7.47 ± 1.852	24.0000 ± 2.85271	39.8667 ± 2.56949	<0.001
Mean difference	-5.267	-13.9000	-37.66667	
t-value	-13.406	-23.722	-45.807	
p-value	<0.001	<0.001	<0.001	

Intra group comparison for cytoplasmic granulation at different time intervals (pre vs during vs post) were done by repeated measures ANOVA

Table 7: Intergroup comparison of leukocytic infiltration score among lesional mucosa and adjacent mucosa by Independent t-test

	Period of study			<i>p</i> -value repeated measures ANOVA
	Pretreatment	During treatment	Post radiotherapy	
Adjacent	30.47 ± 2.569	70.8333 ± 5.29856	100.4333 ± 10.81724	<0.001
Lesional	32.70 ± 8.133	78.4667 ± 17.90537	109.0333 ± 13.17648	<0.001
Mean difference	-2.233	-7.6333	-8.6000	
t-value	-1.434	-2.239	-2.763	
p-value	0.157	0.032	0.008	

Intra group comparison for leukocytic infiltration at different time intervals (pre vs during vs post) were done by repeated measures ANOVA.

All three main treatment modalities-surgery, radiation (RT), and chemotherapy are used to treat oral cancer, either alone or in combination.²⁶

Cellular alterations caused by radiation exposure can be linked to the damage that leads to mitotic cell death. Cell division is initiated and controlled by the centrioles and the pericentriolar matrix (PCM). Radiation-induced peroxidation of lipids in the cell membrane can cause structural and functional alteration to it.²⁷

For oral cancer, cytology has been an option and proved to be a consistent primary diagnostic test. It can also be of value where a surgical biopsy is not indicated or in post-radiotherapy follow-up cases. The combined histological and cytological assessment of a lesion has been found to give the highest percentage of early diagnosis of oral cancers.²⁸

Multinucleation: In the research conducted by Silverman et al., multinucleation was the most common radiation-induced change in oral cancers. These findings were later confirmed by many researchers.¹⁷

Mehrotra and his colleagues observed that in normal mucosa and malignant cells, the frequency of multinucleation was increased with increased

radiotherapy dosage in serial scrape smears from both sides. They also reported a significant association between multinucleation on normal mucosa and malignant cells and radiation dose ($p < 0.001$).²⁹

Before treatment, Bhattathiri et al. observed that the mean multinucleated count was 3.7/1000 mononucleated cells. With radiation, the frequency of cells with multinucleation increased significantly ($p < 0.0001$) to a maximum of 16.8/1000 mononucleated cells.³⁰ Raj et al. in their study found out that multinucleated cells were significantly increased from 0.1 per thousand cells to 0.9 at 24 Gy ($p = 0.05$) on the normal side and from 0.1 per thousand to 1.2 at 24 Gy ($p = 0.01$) on the lesional side.¹⁴

Bhattathiri et al. in their study stated as the fact that multinucleation showed the greatest relationship with radiosensitivity, suggesting that injury to the cytokinetic apparatus is important in determining tumor radiosensitivity.³¹ The findings of our study depict a dose-related change in the number of multinucleated cells which has a highly significant increase in both normal as well as malignant cells. These findings were in accordance with above-mentioned studies.

Nuclear Enlargement

Bindu et al. stated that there was no significant difference in the nuclear shape in relation to radiation dose.¹⁹ Ogden et al. found significant rise in nuclear area resulting from exposure to irradiation (before treatment compared with halfway through treatment).³² Mehrotra et al. in their study observed that as the fractions of the radiotherapy increased, the relative size of the nucleus and nuclear area also increased.³³

An increase in nuclear size after undergoing irradiation was also reported in the present study, a finding which was in accordance with Ogden et al.³² and Mehrotra et al.³³

Cellular Enlargement

Hannah Peters noted an increase in cell size in normal squamous epithelium which received radiation along with cancerous epithelium.³⁴ Ogden et al. found significantly rise in the cellular area resulted from exposure to irradiation (before treatment compared with halfway through treatment).³²

In the present study, the mean value of the lesional and adjacent mucosa showed a significant rise from the pretreatment value to the post-radiotherapy. The results of our study are in accordance with the results of studies conducted by Peters³⁴ and Ogden et al.³²

The results of our study are not in accordance with the study conducted by a study conducted by Silverman et al.¹⁷ He found that in regard to each specific cellular morphologic change, the frequency of enlarged cells increased slightly after the beginning of irradiation. The frequency of cell enlargement showed no consistent relationship to clinical response to neoplasms.

Cytoplasmic Vacuolation

The percentage of the cells showing cytoplasmic vacuolation increased from the pretreatment value of 4.7 to 8.7% in a study conducted by Bindu et al. However, this increase in value was not significant.¹⁹ According to Silverman et al. no change in the degree of vacuolization was observed in patients post radiotherapy.¹⁷ The results of our study were contradictory with the findings of studies done by Bindu et al.¹⁹ and Silverman et al.¹⁷

Agarwal et al. stated that exfoliative cytology of oral squamous cell carcinoma patients undergoing fractionated radiotherapy showed that with increasing dose of radiation, the number of cells exhibiting cytoplasmic vacuolation increased.³⁵ The results of our study are in accordance with this study.

Cytoplasmic Granulation

Hannah Peters stated that the appearance of the cytoplasmic granules in the superficial squamous cells is the most constant microscopic finding after radiation of the squamous epithelium of the mouth.³⁴ According to Mehrotra et al. cytoplasmic granulation has not been reported in previous studies conducted in patients undergoing fractionated radiotherapy. They found that there was a non-specific change in the cytoplasm comprising of cytoplasmic granulation, which was found in both normal and malignant cells. The number was higher in malignant cells and increased with duration of radiation.³³

Mehrotra and his colleagues observed a significant dose-related increase in cytoplasmic granulation count in both normal and malignant cells. The appearance of cytoplasmic granules in the squamous cells is the most constant microscopic finding after irradiation of oral squamous epithelium.²⁹ In the current study, there was a significant dose-related increase in the cytoplasmic granulation in both lesional and adjacent mucosa. The results of our findings were in accordance with above-mentioned studies.

Leukocytic Infiltration

In the present study, the p-value of repeated measure analysis of variance (ANOVA) and post hoc test was statistically significant in both lesional and adjacent mucosa at different time intervals. But on a comparison of the values of adjacent mucosa and lesional mucosa by Independent t-test, the p-value in the pre-treatment smears was not found to be statistically significant.

No significant increase in the extent of neutrophils was observed following radiation exposure in a study conducted by Bindu et al.¹⁹ Agarwal et al. observed that with the increase in dose of radiation there was an increase in the mean percentage of leukocytic infiltration from pre-treatment smear to four weeks post-radiotherapy.³⁵ Ahmed and Elemirri found increased inflammatory infiltrates in post-therapeutic smears, as compared to non-treated patients. This suggests the role of radiotherapy in inducing inflammatory changes.³⁶

Though, among all the parameters considered in the study, leukocytic infiltration was the only finding found, which was not statistically significant in terms of pre-treatment smears on a comparison of the values of adjacent mucosa and lesional mucosa by independent t-test.

The major limitation of this study was sample collection from the unapproachable lesional areas. In addition number of cases is low due to a significant dropout rate of many patients.

CONCLUSION

The findings of the present study emphasize that various nuclear and cellular abnormalities reveal a statistically significant increase with increasing radiation doses and time interval in adjacent normal mucosa as well as lesional mucosa. Knowledge about the sensitivity of the different treatment modalities for oral cancer treatment will greatly benefit judicious treatment planning for these patients. A possible role of the predictive value of these changes deserves further evaluation in a large multi-institutional study.

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