ORIGINAL ARTICLE

Assessment of Various Cytological Changes For Predicting Radiosensitivity of Oral Cavity Cancer by Serial Cytology

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Abstract

A prospective, non-randomized study was carried to assess the role of serial cytological assay in predicting radiosensitivity of squamous cell cancer of oral cavity in patients on fractionated radiotherapy (RT) and to evaluate the relationship of radiosensitivity with the histological grade of oral cancer. We studied 54 histologically proven cases of squamous cell carcinoma on cyclical radiotherapy treatment. Radiosensitivity was assessed using serial scrape smears taken before, during and after treatment with radiotherapy (4 & 8 weeks). Depending on the observed cytological change, patients were grouped in those showing good radiation response (sensitive) and those with poor radiation response (resistant) and their relation to cumulative dose of radiotherapy was analyzed for statistical significance. The changes such as multinucleation, micronucleation, karyorrhexis and cytoplasmic vacuolation occurring in irradiated cancer cells, had a statistically significant dose related increase with radiotherapy ($P < 0.05$). Less differentiated tumors were less radiosensitive and exhibited increased rate of persistence of dysplastic cells and a higher rate of recurrence (33%) after completion of radiotherapy as compared to well differentiated tumors. We recommend regular use of serial cytological assay as it provides valuable evidence of radiosensitivity and persistence of tumor/dysplastic cells at 8 weeks post-radiotherapy.

Key Words

Oral Cancer, Radiosensitivity, Cytology, Radiation, Squamous Cell Carcinoma

Introduction

Carcinoma of the oral cavity is the second most common carcinoma among men after lung cancer in India being predominantly squamous cell in variety in more than 90% of cases (1). Oral carcinoma is mainly treated with radiotherapy alone or in combination with chemotherapy. The response to radiation is determined by the inherent susceptibility of the tumor and its growth rate to radiation damage. Cytological effects of radiation on oral mucosa and in oral cancers were reported in 1957 and 1959 respectively (2). By the 1960s the nuclear morphological changes that were evaluated by cytology became well established and included pyknosis, karyorrhexis, karyolysis, enlargement, multinucleation and crenation of nuclear membrane (3). Both malignant and benign cells show similar changes, although cancer cells showed significant hyperchromasia with increased relative nuclear area, coarse irregularly distributed chromatin and irregular nuclear outlines (4,5). Cellular changes following post-radiotherapy have been described as cytoplasmic vacuolation, nuclear enlargement with clumping of chromatin and wrinkling of nucleus, multinucleation, bizarre cell formation and leukocytic infiltration (6).

Despite a high incidence of oral cancer in South-east Asian region, studies on prediction of effectiveness of various treatment modalities are sparse and inadequate, in this region (7). The present study was undertaken to see whether serial cytological evaluation at 4 and 8 weeks post-radiotherapy, in oral cancer patients of different grades and at different stage can predict oral cancer radiosensitivity or not. Attempts were also made to identify residual tumor cells in scrape smears post-radiotherapy.

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Fig. 1 Acute Radiation Change: Cell Showing Karyorrhexis PAP x500

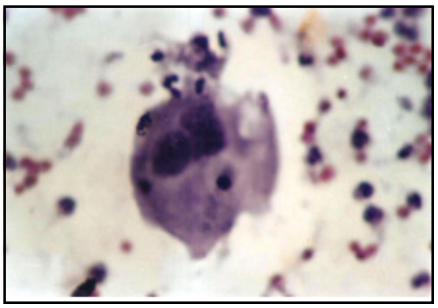

Fig. 2 Acute Radiation Change: Cell Showing Multinucleation PAPx500 Cytoplasmic Changes

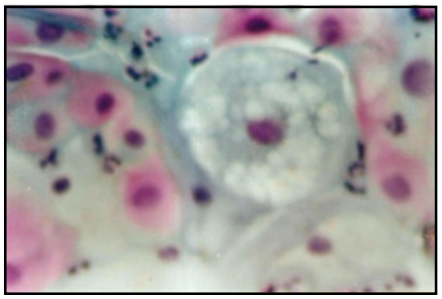

Fig. 3 Acute Radiation Change: Cell Showing Cytoplasmic Vacuolation PAPx500

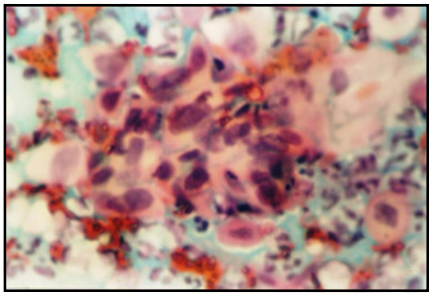

Fig. 4 Recurrence of Squamous Cell Carcinoma :Malignant Squamous Cells with Hyperchromatic Nuclei and Irregular Nuclear Outline PAPx500

Materials and Method

The study was conducted in department of pathology J.N. Medical College, Aligarh Muslim University, Aligarh during the period of two years after approval from ethical review board of our institute. A total of 62 patients with histologically proven squamous cell carcinoma of oral cavity, on fractionated radiotherapy in a dose of 45-55 Gy in 5 fractions/ week for a span of 5 weeks (total 25 fractions), were enrolled in the study. Eight patients were excluded from the study as did not turned for follow up at various intervals after submitting pre-radiotherapy scrape smear, therefore limiting the sample size to 54 patients.

Scrape smears were collected from tumor area of each patient before the start of treatment. Of the total 25 fractions of radiotherapy received by cases, smears were collected on 8th and 11th fraction of treatment and at 4 and 8 weeks interval post-completion of radiotherapy. The material was obtained by scraping the lesion using the rounded end of Ayre's spatula and slides were fixed in 95% alcohol for papanicolaou (Pap) stain, while some slides were air dried for May-Grunwald-Geimsa (MGG) stain. Smears were examined at both 40x and 100x with an eyepiece of 10x.

Around 500-1000 cells were evaluated from the samples collected on each occasion. The nuclear and cytoplasmic changes observed were cytoplasmic vacuolation, multinucleation, wrinkling of nuclei, nuclear budding and karyorrhexis along with leukocytic infiltration. Binucleate cells were not included in this because these could be cells undergoing normal mitosis. The change in the frequency of these changes in relation to cumulative radiation dose was analyzed using chi-square test/ Fisher exact test and a P-value < 0.05 was considered as statistically significant. The patients were grouped to (1) sensitive group, showing good radiation response and (2) resistant group, with poor response and with persistence of dysplastic or malignant cells, following the radiotherapy. Response to radiotherapy was considered good if greater than 75% of cells showed radiation changes, mild if 60-75% showed changes and poor (dysplastic) if $< 60\%$ showed changes

Results

The clinical and demographic data of patient population (54 patients) is shown in *Table 1*. Addiction to tobacco smoking or chewing was present in all 54 patients with 43 patients being addicted to both and 11 patients to either smoking or tobacco chewing.The radiation response was considered to be good in 23 patients (71.87%) with well differentiated Ca, 11 patients (68.75%) with moderately differentiated carcinoma and only 3 patients (50 %) with

Table 1. Demographic Data of Patients

Age	Number of patients (54)
Range (in years)	27-65
[Mean ± SD (range)] in years	45.6± 6.8
History of smoking	
Non smokers	8
Smokers	46
History of Tobacco Chewing	
Tobacco chewer	45
Non tobacco chewer	9
Staging (AJCC)	
I	24
II	10
III	18
IV	2

poorly differentiated carcinoma at 8 weeks post radiotherapy, thus indicating a strong relationship between tumor grade and radiosensitivity (*Table 2*): The following cytological parameters were observed in this study:

Nuclear changes: Karyorrhexis signifies nuclear breakup into smaller fragments (*Fig. 1*) while karyolysis signifies a progressive dissolution of chromatin.

Nuclear buds represent the rounded nuclear material mimicking a micronucleus and can often be found close to the nucleus without any definite separation. Micronuclei are intracytoplasmic, DNA staining bodies found in the same plane as the main nucleus with the same or slightly lesser staining intensity, one-third to one-fifth of the size of the main nucleus, placed within two nuclear diameters from the main nucleus, but distinctly separate from it. Multinucleation (*Fig 2*) is caused by membrane damage associated with accelerated proliferation of the nucleus, resulting in an inability of the membrane to keep up with the nuclear division.

Table 2. Correlation of the Degree of Radiation Response with Grade of Squamous Cell Carcinoma of Oral Cavity

Histological Grade	No of patients	Radiation Response (8 weeks post radiotherapy)		
		Good (%)	Mild (%)	Dysplastic (%)
Well Differentiated	32	23 (71.87)	9 (28.12)	0 (0)
Moderately Differentiated	16	11 (68.75)	4 (25)	1 (6.25)
Poorly Differentiated	06	3 (50)	1 (16.6)	2 (33.3)
Total	54	37 (68.51)	14 (25.92)	3 (5.56)

Table 3. Various Cytological Changes Seen Pretreatment, During & Post-Radiotherapy with their Statistical Significance

Cytological changes	Mean percentage (%) of cellular changes					
	Pre-treatment	8th cycle (28Gy)	11th cycle (38.5Gy)	4 weeks	8 weeks	P-value
Cytoplasmic vacuolation	4.1	7.1	8.1	8.7	9.8	0.012
Leucocytic infiltration	6.1	8.8	9.1	10.4	9.8	0.042
Micronuclei	1.4	5.8	6.4	7.1	7.7	<0.001
Karyorrhexis	0.4	2.3	2.8	3.3	3.9	0.01
Multinucleation	0.8	4.4	5.7	6.4	6.9	<0.001
Nuclear budding	0.6	1.1	1.7	2.3	2.7	0.034

Table 4. Correlation of Degree of Radiation Changes with time Interval following Radiotherapy

Time interval	No of patients	Radiation Response		
		Good (%)	Mild (%)	Dysplastic (%)
During radiotherapy	54	16 (29.6)	22 (40.74)	16 (29.62)
4 week post-radiation	54	30 (55.56)	21(38.8)	3 (5.56)
8 week post-radiation	54	37 (68.51)	14 (25.92)	3 (5.56)

Cytoplasmic vacuolation (*Fig 3*) is one of the earliest changes as a result of radiation exposure seen at the light microscopic level. The number of cells showing this change increased with increasing dose of radiation. Some squamous epithelial cells contain a few membrane coated granules called keratohyaline bodies. Presence of leucocytes is a common phenomenon in irradiation smears. It is the close association of neutrophils superimposed onto the squamous cells. In many cases, neutrophils may be so numerous as to obscure the true nature of the underlying cell. All these nuclear and cytoplasmic changes increased with radiation dose, but were found to be statistically significant only when these changes persisted even after 8 weeks of completion of radiotherapy (P=0.042) (*Table 3*). The response to radiotherapy showed cytological improvement with increasing time interval, the changes most appreciated at 8 weeks interval post-radiotherapy (*Table 4*). It was seen that well differentiated tumors responded better to radiotherapy with less frequency of recurrence as compared to poorly differentiated tumors. Increased chance of recurrence is indicated by presence of dysplastic cells, hyperchromatic nuclei, irregular nuclear outline, small dark tumor cells or large naked ovoid nuclei especially at 8 weeks after completion of radiotherapy (*Fig. 4*).

Discussion

The present study aimed to observe the cytological changes following radiation (radiation reaction) and predict the strength of relationship between dose and duration of radiation therapy to these changes. Silverman et al. reported multinucleation as commonest radiation-induced change in oral cancers which was later confirmed by other researchers, but none of them established any correlation between radiation induced changes and the radio-sensitivity of tumor cells (8,9). In these studies, the changes were evaluated only in benign cells collected from normal mucosa around the tumour, excluding any malignant cells. Our results showed that various quantifiable cellular changes became evident in the initial few days of radiotherapy but are more prominent at 8 weeks post-radiation, when they show strong statistical significance.

Cell division is complete when the division of cell membrane occurs following nuclear division. Incomplete cell division occurs due to cell membrane damage which leads to the formation of binucleated and multinucleated cells by repeated nuclear division. Membrane lipids can undergo peroxidation following irradiation and this damage may be sufficient to prevent cell wall division in sensitive cells (9,10). Radiation induced multinucleation has been noted in animals and cell culture experiments. Damage to nuclear membrane has been postulated as a mechanism that leads to cell death, therefore multinucleated cells are considered to be dead cells and incapable of giving rise to colonies(11). This along with high intratumoral variability suggests that multinucleated cells can prove to be a useful diagnostic tool in predicting radiosensitivity. Nuclear blebbing can occur in the dynamic nuclear envelope and its rupture leads to transfer of DNA material to cytoplasm, therefore nuclear budding can be considered to be induced by a direct, localized effect of radiation on nuclear membrane(10,12).

Micronucleation assay in cultured and irradiated tumor cells has also been evaluated as a sole predictive test for radiosensitivity by some authors, though the results have been more reliable when all the cellular changes are evaluated simultaneously (12,14). Micronuclei are eccentric, chromosomal fragments that lag behind during mitosis and fail to be incorporated into the main nucleus of daughter cells after cell division. The presence of micronucleus indicates that the cell has suffered unrepaired DNA damage and micronuclei cells are considered dead cells incapable of giving rise to progeny (15). The presence of a micronucleus is an accepted test for monitoring toxicity of chemicals and effectiveness of chemopreventive agents against cancer (16). The number of micronucleated cells increase and reach a plateau with repeated chemical and radiation injuries (12). Similar finding was reflected in our study with the micronuclei increasing with radiation dose. Earlier studies with micronucleation and multinucleation assays during the first 15-18 days of radiotherapy showed serial cytology to have significant and important correlations with radio-sensitivity and cell proliferation (7,14,17).

We observed that when different parameters (multinucleated cells, nuclear budding, micronucleation and bizarre cells) were taken together as one, the increase in dose related response was significantly high. These abnormally nucleated cells had a mean value four times higher than pretreatment count at 38.5 Gy, thus indicating this combined parameter to be a better indicator of radiosensitivity than any single parameter. In concordance with other studies, we observed that well differentiated tumors in initial stage responded better to radiotherapy than less differentiated ones or those presenting in advanced stage (8,13,17). Poorly differentiated carcinoma

had higher incidence of recurrence (33.3%) and poor radiological response as compared to moderately (6.7%) and well differentiated squamous cell carcinoma (0%) in our study. Similar findings were observed by Crissman *et al*, Frieson *et al* and Rimpu *et al*. (18,19,20).

In our study, 7 (12.9%) patients showed a shift in radiation response from mild at 4 weeks to good response at 8 weeks. We also observed that presence of dysplastic or malignant cells in smears prepared at 4 weeks post-radiotherapy was not significant. But cases with dysplastic cells visualized at 8 weeks post-radiotherapy had either resistance to therapy or developed local recurrence later on. Previous studies have tried to establish the role of single cellular change (multinucleation, micronucleation etc.) in predicting radiotherapy response at various phases of treatment. Our study showed that collective count (termed as abnormally nucleated cells) was stronger predictor of radiation induced changes in malignant squamous cells. Moreover, as compared to all the few previous studies which evaluated the cellular changes at 2 to 4 weeks post-treatment, our study showed that final evaluation of radiation response in oral cancers, is more accurate and reliable after 8 weeks post-radiotherapy.

Conclusion

It is thus concluded that various nuclear and cytoplasmic abnormalities demonstrate a statistically significant increase with radiation dose and time interval. Smears taken during and immediately after radiation therapy were of little prognostic significance as both malignant and benign cells showed similar changes, but after 8 weeks of completion of radiotherapy these cellular changes were highly significant. Persistence of dysplastic and malignant cells during and beyond this period may indicate resistant or recurrent carcinoma. The response to radiotherapy was more prominent in low grade tumors (well differentiated) as compared less differentiated tumors (higher grade). We thus, recommend serial cytological assay of tumor area till 8 weeks post-completion of radiotherapy, as a potential tool for prediction of radiosensitivity.

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