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Cytokinetic Studies of Oral Cancer Cells Using Bromodeoxyuridine Labelling in Relation to Factors Influencing Prognosis

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Cytokinetics of 27 untreated oral squamous cell carcinomas were evaluated by *in vitro* bromodeoxyuridine (BrdU) labelling. A mononuclear cell suspension was prepared for the labelling using collagenase and DNase treatment. The labelled cells were visualised by immunofluorescence staining with an anti-BrdU monoclonal antibody. The labelling index (LI%) was calculated by determining the percentage of BrdU labelled cells. The LIs of the carcinomas ranged from 3.8 to 19.2%. The LIs of poorly-differentiated (HG3) tumours were 2–3-fold higher compared with those of well-differentiated (HG1) and moderately-differentiated (HG2) carcinomas. Results also showed statistically significant ($P<0.0005$) increases in LIs from nuclear grade 1 (NG1) to nuclear grade 3 (NG3) carcinomas. Higher LIs were observed in stage III/IV (10.82 ± 4.62; 10.36 ± 4.90) than those in stage I (6.87 ± 2.09) and II (7.14 ± 1.87) carcinomas. A significant ($P<0.0005$) difference in LI values was found between the patients with positive and negative lymph nodes. Good correlation ($r=0.77$) was exhibited between the LI values and mitotic counts (MC) of the specimens. These results on oral cancer cell proliferation seem to have prognostic implications.

Keywords: HPV, carcinoma, squamous cell, bromodeoxyuridine, oral cancer, cell division, neoplastic

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INTRODUCTION

SEVERAL REPORTS [1–3] have shown that the study of human tumour cell kinetics may be of value in understanding neoplastic growth. A knowledge of the cell proliferative status of tumours is indicative of their aggressiveness, sensitivity to antineoplastic agents and is also useful for prognosis [4–7].

Tumour cell kinetics are well studied by a recently developed method using *in vivo* [6, 8–11] and *in vitro* [3, 4, 12] bromodeoxyuridine (BrdU) labelling of cells. BrdU, a thymidine analogue which is incorporated into DNA synthesising cells (S-phase) can be detected using a monoclonal antibody against BrdU [13, 14]. The non-radioactive BrdU method of analysing cell kinetics has been found to be not only faster than the autoradiographic procedure [15, 16] but also equally competent, as the two techniques showed good correlation [17]. This technique has been followed extensively for cytokinetic investigations of brain tumours [4, 18], cervical tumours [19], pulmonary adenocarcinomas [20], tumours of head and neck [8, 21], lung cancer [22], hypopharyngeal and oropharyngeal cancer [9, 23], breast cancer [24], squamous cell carcinomas of oral cavity [12] and many other types of tumours [10, 11, 25].

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In this paper, we describe assessment of the presence of cells in S-phase in oral cancer lesions with different histological grading. BrdU incorporation *in vitro* was monitored using immunofluorescence. The proliferative activity was analysed in relation to other factors, namely clinical stage, nuclear grade and mitotic activity, that are important for prognosis. In the present study we sought to evaluate the relationship between tumour cell proliferation of squamous cell carcinomas of the oral cavity and the factors influencing prognosis.

PATIENTS AND METHODS

Patients

Out of the 49 oral cancer patients treated at the Chittaranjan Cancer Hospital, Calcutta, between June 1992 and May 1993, 27 were randomly selected for this investigation with squamous cell carcinomas at different sites of oral cavity (six cases of carcinoma of the left and four of the right cheek; two cases of carcinoma involving the jaw and left cheek; five of lower alveolar carcinoma; three cases of tongue cancer in the lateral margin and one at the lower surface of tongue; three with cancer of the hard palate; two with cancer of the floor of the mouth; one carcinoma of the left jaw). The specimens studied were obtained either surgically or by punch biopsy from the patients prior to the start of their treatment. The age of the patients (23 men and 4 women) ranged from 35 to 65 years (average age, 52 years).

Histopathology and mitotic counts

Histological study using haematoxylin–eosin staining revealed that 11 cases were well-differentiated (HG1), 13 moderately-differentiated (HG2) and three poorly-differentiated (HG3) squamous cell carcinomas. The criteria followed for these differentiations were as defined previously [26]. For well-differentiated tumours the observed features were dysplasia, hyperchromatism, plemorphism, increased nuclear to cytoplasm ratio, cell nests, keratin pearls and premature keratinisation. In moderately-differentiated carcinomas cell nests were absent although keratinisation of cells were observed. However, obvious keratinisation was not evident in the poorly-differentiated tumours.

The stained sections were further examined for grouping the specimens on the basis of nuclear grading [27]. Specimens showing few mitoses and well-differentiated cells with elongated or oval nuclei, very small nucleoli and fine chromatin pattern were graded NG1; those having round enlarged nuclei, large multiple nucleoli and a number of mitoses were termed as grade 3 (NG3). The specimens having nuclear changes more than NG1 but less than NG3 were classified as NG2. Mitotic counts per 10 high power fields (corresponding to 2.5 mm^2) were obtained by examining the stained histologic sections of the specimens under the microscope.

TNM staging

The malignancies were also classified into stage I (T1), II (T2), III (T3) and IV (T4) according to the American Joint Committee for staging of cancer [28].

BrdU incorporation and visualisation

To study *in vitro* BrdU incorporation, the method of Teodori *et al.* [15] was followed. The chemicals used were obtained from Sigma, St Louis, Missouri, U.S.A. Minced fresh tissue was suspended in RPMI 1640 medium containing nonessential amino acids (Gibco, U.S.A.), 10% fetal bovine serum, 0.25% collagenase IA, 0.01% DNase, 30 mM BrdU and 30 mM deoxycytidine. The suspension was then incubated (in dark) for 1 h in a shaker bath at 37°C.

The harvested cells were rapidly rinsed with cold PBS (0.01 M, pH 7.5) and then fixed in 70% ethanol for 10 min. Cells were washed in PBS–0.1 N EDTA; resuspended in 3 N HCl for 40 min and then 0.1 N sodium borate solution was added. Cells washed with PBS–EDTA and 0.5% Tween-20 were treated with the anti-BrdU monoclonal antibody (Clone BMC 9318; Boehringer-Mannheim) for 1 h. Cells were washed after removing the antibody and treated with fluorescein isothiocyanate (FITC)-labelled goat antimouse IgG. The cells were then examined by fluorescence microscopy. At least 500 cells were counted. The labelling index (LI) was determined by observing the number of BrdU labelled nuclei and then expressed as a percentage of the total number of nuclei examined.

The oral epithelial cells were distinguished from connective tissue and inflammatory cells by an immunohistochemical method [29] using antibodies to cytokeratin, desmin, vimentin and leukocyte common antigen (LCA). The epithelial cells tested positive for only cytokeratin and were negative for tests with the other antibodies. Positive reactions for vimentin and desmin were characteristic for the connective tissue and that with the LCA were unique for inflammatory cells.

Statistical analysis

Student's *t*-test was performed to determine any significant difference in LI values between (a) HG3 and HG 1/2; (b) NG1 and NG3 groups of oral carcinomas and also (c) between the oral tumours with or without involvement of lymph nodes. To investigate any significant correlation between LI values and mitotic counts (MC) of the carcinomas Pearson's correlation test was used.

RESULTS

A representative BrdU labelled oral cancer cell is illustrated in Fig. 1. The FITC-fluorescence was observed to be localised mainly in the nucleus. Figure 2 shows characteristic features of the different nuclear grades of carcinomas as observed under light microscope.

Table 1 shows the LI values as obtained by the BrdU method for all the 27 specimens tested, their histological and nuclear grades, TNM staging and mitotic counts. Homogeneity in the cytokinetic (LI%) distribution was observed in the oral carcinomas of the same histological grades in all three groups (HG1, HG2 and HG3). A variation in the LI values (mean) of the carcinomas at different sites of the oral cavity was observed.

Mean LIs and mitotic counts together with the range of values are reported in Table 2 according to histological and nuclear grading of the lesions. The LIs of well-differentiated (HG1) oral carcinomas ranged from 3.8 to 9.5% ($6.59 \pm 1.76\%$; mean $\pm$ S.D.) and a large number (seven of eleven) of them had values below 7%. In contrast, most (twelve of thirteen) of the moderately-differentiated (HG2) type had LI values 7% or above. The LI values ranged from 5 to 10.6% ($8.56 \pm 1.58\%$). Results showed that HG2 carcinomas had significantly higher ($P < 0.0005$) LI values compared to HG1 type. Significant differences ($P < 0.0005$) in LIs between poorly-differentiated (HG3) and the other two types (HG1 and HG2) of carcinomas were observed. Thus, LI values tended to increase at higher grades, that is less differentiated form of carcinomas.

The oral carcinomas of higher nuclear grades exhibited greater LI values (Table 2). The differences in LIs between the NG1 and NG2 types of carcinomas and also that between the NG2 and NG3 types were statistically significant ($P < 0.0005$).

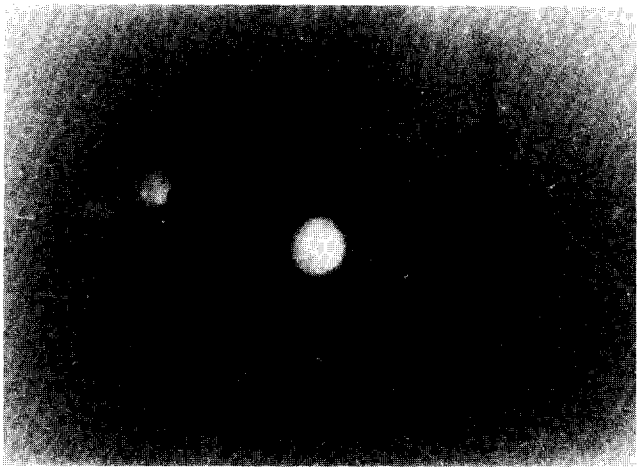

Fig. 1. A representative example of a BrdU-labelled oral cancer cell, × 400.

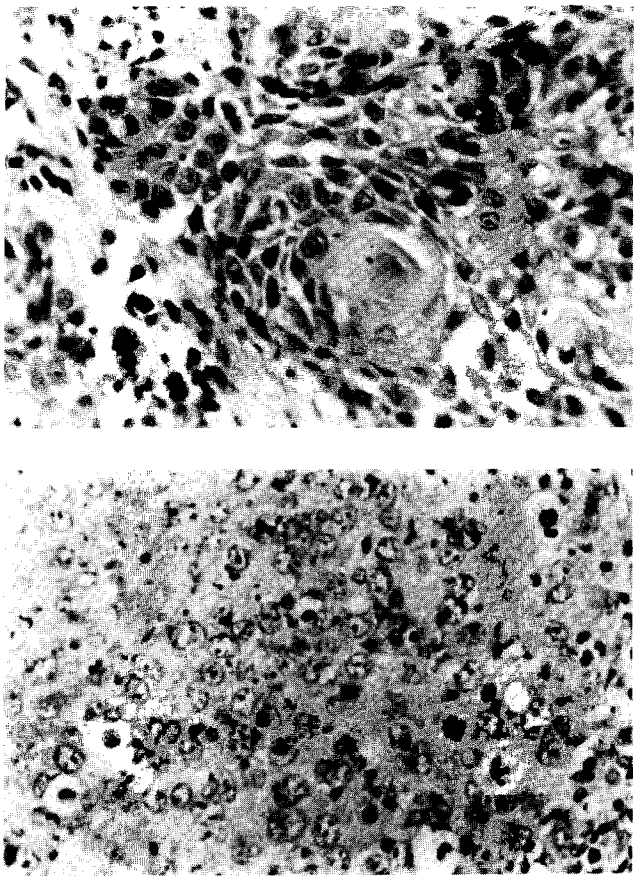

Fig. 2. Photographs of haematoxylin-eosin (× 500) stained sections. (a) nuclear grade 1 (NG1); (b) nuclear grade 3 (NG3).

Results presented in Table 3 revealed a trend of increasingly high LIs for the oral tumours at higher TNM stage. However, the differences in mean LI values of the groups of carcinomas at various stages (I–IV) were not significant. The mean LIs of the oral lesions (14 cases) with positive lymph nodes ($N_1 + N_2$) was 10.9 ($\pm 4.25\%$; S.D.) and that for the lesions (11 cases) with negative lymph nodes (N_0) was 7.04 ($\pm 1.85\%$; S.D.). The difference in the LI values between these two groups was statistically significant ($P < 0.0005$). The node positivity was defined as the presence of metastasis of the squamous cell carcinomas in the lymph nodes.

The mitotic counts (MC) per 10 high power fields (HPF) (× 400 magnification) as obtained during a histological study of the tissues were compared with the LI values. The mean values of the mitotic counts (MC) were found (Table 2) to increase in the oral carcinomas with increasing anaplasia from the well- (HG1) to less-differentiated form and also from NG1 to NG3 grades. Identical variations in the LI values were also observed (Table 2).

The MC of the oral carcinomas studied, as listed in Table 1, ranged from 0 to 43 per 10 HPF ($23.74 \pm 12.09\%$). The lowest (0) MC was obtained in an HG2 type carcinoma with LI 7.4%. The highest (43) MC was observed in an HG3 type carcinoma having a LI value 18.6%. Thus, increased LI values were obtained with increasing MC suggesting a correlation ($r = 0.77$, $P < 0.001$) between these two variables (Fig. 3). The oral carcinomas with LI values less than 8% had MC ranging between 7 and 22. But cases with higher LIs (8–19.2%) had an increased MC (from 22 to 43). The pattern of variations in the MC (mean) (Table 1) with the sites of the oral cavity affected by cancer was also similar to the corresponding variations in the mean LI values.

DISCUSSION

In the present study we determined the proliferative cell index of 27 cases of squamous cell carcinomas at different sites of oral cavity. Their proliferative behaviour was analysed in relation to different prognostic factors such as histologic and cytologic changes and TNM stages.

In the current investigation LIs tended to increase from HG1 to HG3 grades of the oral carcinomas suggesting an association of higher LIs with the less differentiated type of cancer. Similar observations have been reported previously in studies with human endometrium [30], lung cancer [22], hypopharyngeal carcinoma [23] and cerebral astrocytic tumours [17]. Pronounced proliferative activities (higher LIs) were observed by the authors in all the poorly-differentiated (HG3) squamous cell carcinomas, the aggressive form of the malignant tumours.

Results of this study also showed that the cell kinetics tended to increase with higher nuclear grade of the oral cancer lesions tested. This correlates with high proliferative cell index in endometrial adenocarcinomas of higher nuclear grade, as reported previously [31]. Nuclear grading was performed by the authors on the basis of characteristics of the nuclei and nature of mitoses present in the lesions. An earlier study [32] on pulmonary adenocarcinoma indicated that aberrations of nuclear areas of tumour cells may correlate with their nuclear pleomorphism or atypia. Hence, the nuclear grading may be indicative of the nuclear atypias of the tumour cells. A greater number of cells in the DNA synthesis phase (S-phase) were obtained at a higher degree of nuclear atypia in a study of pulmonary adenocarcinoma by the BrdU method [20]. This observation concurs with our findings.

Good correlation between BrdU LIs and TNM stage classification of tumours has been demonstrated during studies on the proliferative rate of pulmonary adenocarcinomas [20], carcinomas of the larynx [33], and carcinomas of the oral cavity [12]. However, a previous cytokinetic investigation of lung tumours found no correlation between LI and TNM staging [15]. In our study, higher LIs (though not significant) were found for stage III/IV than for the stage I/II carcinomas. Thus, the cases with poor prognosis (stage III/IV) had cytokinetics apparently higher than that of the cases with better prognosis (stage I/II). We also found significant difference in LI values between the cases with and without positive lymph nodes. This was contradictory to the observations of the authors [33] who worked on ^{3}H-thymidine LIs in squamous cell carcinomas of the larynx.

The mitotic index has been studied to characterise growth of solid tumours. Such a study has been correlated with the prognosis [32, 34]. BrdU LI has been shown previously to correlate with mitotic cell counting (MC) [20, 35]. Similar results were obtained in the present study.

In conclusion, this study of *in vitro* incorporation of BrdU provides interesting information about the cell growth of squamous cell carcinomas of the oral cavity. The results indicate that LI varies directly with the dedifferentiation process and metastasis of the oral carcinomas. They also appear to have prognostic implications.

Table 1. Histological and TNM classification, LI and MC of the specimens studied

Patient No. (site of cancer)	Grade		TNM/Stage	LI%	Mean LI% (site-wise cancer)	Mitotic Count	Mean Mitotic Count (site-wise cancer)
	Histological	Nuclear					
Ca-cheek							
1	HG1	NG2	$T_2N_0M_0$/II	6.0	10.34	28	28.7
2	HG1	NG2	$T_2N_0M_0$/II	6.8	—	15	—
3	HG1	NG1	$T_1N_0M_0$/I	4.6	—	13	—
4	HG2	NG2	$T_2N_1M_0$/III	10.6	—	34	—
5	HG2	NG2	$T_3N_2M_1$/IV	8.4	—	22	—
6	HG2	NG2	$T_3N_1M_0$/III	10.6	—	31	—
7	HG2	NG2	$T_4N_1M_0$/IV	8.6	—	33	—
8	HG3	NG3	$T_3N_1M_0$/III	18.6	—	43	—
9	HG3	NG3	$T_2N_2M_0$/IV	19.2	—	39	—
10	HG2	NG2	$T_2N_1M_0$/III	10.0	—	29	—
Ca-cheek and jaw							
11	HG1	NG1	$T_2N_1M_0$/III	5.4	7.2	7	15.0
12	HG1	NG2	$T_2N_0M_0$/II	9.0	—	23	—
Ca-alveolus							
13	HG1	NG1	$T_2N_0M_0$/II	3.8	6.5	5	12.8
14	HG1	NG1	$T_1N_0M_0$/I	6.0	—	10	—
15	HG1	NG1	$T_2N_0M_0$/II	7.0	—	11	—
16	HG1	NG1	N.A.	6.2	—	9	—
17	HG1	NG1	$T_1N_0M_0$/I	9.5	—	29	—
Ca-tongue							
18	HG2	NG2	$T_2N_0M_0$/II	8.4	8.3	27	23.2
19	HG2	NG2	$T_1N_1M_0$/III	8.4	—	31	—
20	HG2	NG2	$T_1N_0M_0$/I	7.4	—	0	—
21	HG2	NG2	$T_2N_0M_0$/II	9.0	—	35	—
Ca-hard palate							
22	HG2	NG2	$T_2N_2M_0$/IV	7.6	7.33	42	24.7
23	HG2	NG2	$T_3N_1M_0$/III	9.4	—	24	—
24	HG2	NG1	N.A.	5.0	—	8	—
Ca-floor of mouth							
25	HG1	NG2	$T_3N_1M_0$/III	8.2	12.3	32	34.0
26	HG3	NG3	$T_3N_1M_0$/III	16.4	—	36	—
Ca-jaw							
27	HG2	NG2	$T_3N_2M_0$/IV	8.0	N.A.	25	N.A.

N.A., not available.

Table 2. LI% and mitotic counts in the oral squamous cell carcinomas according to histological and nuclear grading

Group	n	LI%		Mitotic Counts	
		Mean ± S.D.	Range	Mean ± S.D.	Range
HG1	11	6.59 ± 1.76	3.8–9.5	16.54 ± 9.67	7–32
HG2	13	8.56 ± 1.58*	5.0–10.6	26.23 ± 11.29	0–42
HG3	3	18.06 ± 1.47*	16.4–19.2	39.33 ± 3.51	36–43
NG1	8	5.93 ± 1.74	3.8–9.5	11.50 ± 7.48	7–29
NG2	16	8.52 ± 1.26**	6.0–10.6	26.93 ± 9.53	0–42
NG3	3	18.06 ± 1.47**	16.4–19.2	39.33 ± 3.51	36–43

*$P < 0.0005$ (HG2 versus HG1; HG3 versus HG1 and HG2).
**$P < 0.0005$ (NG2 versus NG1; NG3 versus NG2).

Table 3. TNM stage of the oral cancer cases and BrdU labelling indices (LI%)

TNM stage	*n*	LI% Mean ± S.D.	LI% Range
I	4	6.87 ± 2.09	4.6–9.5
II	7	7.14 ± 1.87	3.8–19.2
III	9	10.82 ± 4.62	8.0–18.6
IV	5	10.36 ± 4.90	7.6–19.2

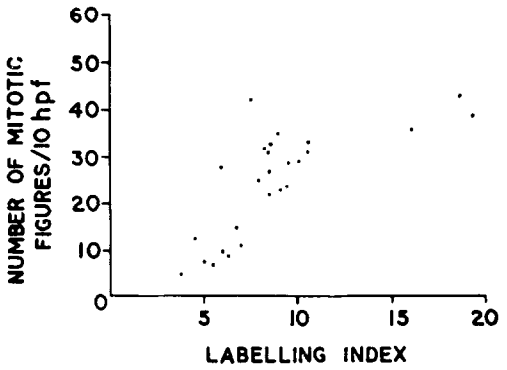

Fig. 3. Relation between mitotic counts (MC) and labelling indices of the oral carcinomas.

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