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Flow Cytometric DNA Analysis of Squamous Cell Carcinomas of the Oral Cavity: Correlation with Clinical and Histopathological Features

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DNA content was measured in 68 squamous cell carcinomas (SCC) of the oral cavity by flow cytometry. Samples fixed in 95% alcohol and disaggregated with 0.5% pepsin were stained with 4,6-diamidino-phenylindole (DAPI) for flow cytometry. The tumours were classified according to the TNM classification—1987, and graded histopathologically. A positive correlation between tumour size and ploidy status was observed. Poorly differentiated tumours were mainly non-diploid ($P < 0.01$, χ^2). A majority of the node positive (N+) tumours were non-diploid ($P < 0.05$). It was possible to distinguish diploid, N+, T4 tumours from diploid, N−, T4 tumours by their higher S-phase fraction (SpF). SpF was also a useful parameter to differentiate diploid, N+ tumours from diploid, N− tumours among the moderately differentiated (MD) SCC. These results suggest that ploidy and SpF can be useful correlates of tumour behaviour.
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INTRODUCTION

SQUAMOUS CELL carcinomas of the oral cavity form a major group of malignancies in India [1] and are responsible for considerable morbidity and mortality. The TNM classification [2] and malignancy grading [3] are generally useful as prognostic aids and for planning therapy. It has been observed that tumours of the same grade/stage do not necessarily have the same clinical course. It is, therefore essential to identify additional biological correlates of tumour behaviour. Measurement of cellular DNA content by flow cytometry gives the ploidy and proliferative status of tumours. Numerous workers have correlated DNA ploidy and proliferative status with: clinicopathological features, response to therapy, and prognosis, in various tumours namely, those of breast, bladder, lung, uterine-cervix, colorectum and kidney [4, 5]. Such a correlation has been substantiated in some studies but not in others [4, 5]. We have carried out flow cytometric measurements on the DNA content of oral tumours to evaluate its relation, if any, with clinicopathological features.

MATERIALS AND METHODS

68 squamous cell carcinomas (SCC) of the oral cavity were analysed. These consisted of 37 SCC of the buccal mucosa and 9 of the tongue. The rest were from other sites, such as soft palate, floor of the mouth, lower jaw and maxilla. The age of the patients ranged from 35 to 70 years. There were 61 males and 7 females.

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Sample preparation

Tumour samples were collected during surgery in Dulbecco's modified Eagle's medium (DMEM). After removing blood and necrotic tissue, the sample was cut into small pieces, fixed in 95% ethanol and stored at 4°C until used for flow cytometric measurements.

The fixed tumour pieces were further cut into smaller pieces and disaggregated with 0.5% pepsin in normal saline (pH 1.5) for about 20 min at room temperature. Nuclear DNA was stained with DAPI such that the ratio of pepsin to DAPI was 1:4 and the final cell concentration was $0.5–1.0 \times 10^6$ cells/ml [6]. The suspension was filtered through a 50 μ nylon mesh and passed once through 26 gauge needle just before measurements. Measurements were done 30 min after staining.

Ficoll–Hypaque separated human peripheral blood lymphocytes were used as a calibration standard [7]. They were fixed in 70% ethanol and stained as above.

Flow cytometry

Flow cytometric analysis was done on a PAS-II (Partec, GmbH, Germany) flow cytometer fitted with a HBO-100 mercury lamp. The filters used were KG1 + BG38 and UG1, dichroic mirrors—TK-420, TK-560 and barrier filter GG 435. The instrument was adjusted for a coefficient of variation (CV) between 1.1 and 1.4 for human lymphocytes. The CV of

the lymphocytes and of the G0/G1 population of the tumour sample was calculated according to Dean *et al.* [8]. Data from 25 000 cells per sample was collected in all cases.

The DNA Index (DI) of the G0/G1 peak was calculated as the ratio of the modal channel of the G0/G1 peak of the tumour sample to the modal channel of the lymphocytes. Cell cycle parameters, i.e. the percentage of cells in G1 and in S+G2+M—proliferation index (PI)—and the S-phase fraction (SpF) were calculated using a software provided by the manufacturer. The SpF was calculated only in case of unimodal diploid histograms, where there was no overlap of two or more modes.

The histograms obtained were considered to have DNA aneuploidy only when at least two separate G0/G1 peaks were seen [9]. Unimodal histograms with only one G0/G1 peak and with DI of 0.85–1.15 were considered diploid (Fig. 1a). The sample was considered tetraploid when G2+M (4c DNA) peak formed at least 25% of the total cells and a discrete peak at 8c was present (Fig. 1b). The aneuploid tumours were classified as bimodal when they had one diploid and one aneuploid stem line (Fig. 1c). They were considered multimodal if they had one diploid and two aneuploid stem lines (Fig. 1d). Aneuploid and tetraploid tumours have been grouped together as non-diploid tumours for analysis.

Correlation between various parameters was analysed by the χ^2 test.

RESULTS

The distribution of tumours according to TNM classification—1987 [2], histopathological grade [3], and cervical lymph node metastasis (CLNM) is given in Table 1. The T-status was not available for 5 patients. The CLNM status (N+, node positive; N−, node negative) of one T4 tumour was not known. None of the patients had distant metastasis.

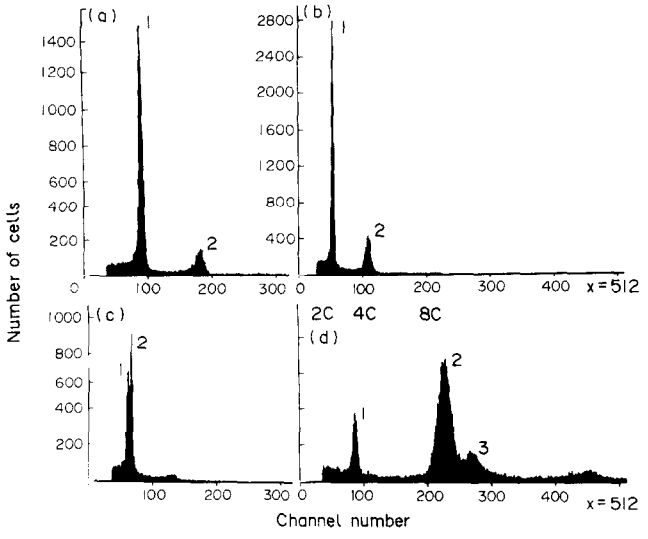

Fig. 1. Representative DNA histograms of human oral tumours. Abscissa—Channel number showing relative DNA content. Ordinate—Number of cells. (a) Diploid tumour from SCC of tongue. Peak 1: DI=0.9, CV=3.2, SpF=20.6%. (b) Tetraploid tumour from SCC of buccal mucosa. Peak 1: DI=1.0, CV=3.9; peak 2: DI=2.0, CV=3.1. A small peak at 8c seen. (c) Aneuploid bimodal tumour from SCC of buccal mucosa. Peak 1: DI=0.9; peak 2: DI=1.0. (d) Aneuploid multimodal tumour from SCC of maxilla. Peak 1: DI=0.9, CV=3.4; peak 2: DI=2.4; Peak 3: DI=2.9.

Table 1. Distribution of oral tumours with reference to size, grade and cervical lymph node metastasis

Tumours (*n*)	Size	Grade I	Grade II	Grade III	CLNM Status N+	CLNM Status N−
1	T1	1	—	—	—	1
10	T2	3	5	2	3	7
10	T3	3	5	2	6	4
42	T4	11	24	7	25	16
Total 63*		18	34	11	34	28

*Clinical staging not available for 5 patients.

Table 2. Ploidy distribution among oral tumours of different sizes

Size (*n*)	Diploid	Non-diploid
T1 (1)	—	1
T2 (10)	9 (90%)	1 (10%)
T3 (10)	4 (40%)	6 (60%)
T4 (42)	20 (47.6%)	22 (52.3%)

χ^2; $P<0.05$.

Out of 68 tumours, 39 (57.3%) were diploid, 21 tumours (30.8%) were aneuploid and eight tumours (11.7%) were tetraploid. The DI values in the tumours ranged from 0.5 to 2.3. The SpF was calculated for 45 tumours (67%) and ranged from 2.9% to 33.0% (Mean±S.D. 17.7%±6.5). In seven tumours, the SpF was below 10%; 24 tumours had SpF between 10 and 20% and 14 tumours had SpF higher than 25%. There was no direct relation between DI and SpF.

The percentage of diploid and non-diploid tumours in relation to the tumour size is shown in Table 2. A majority of the T2 tumours (90%) were diploid. The DI values among the T2, T3 and T4 tumours are shown in Fig. 2. The DI values of T3 tumours ranged from 0.5 to 1.8 and of T4 tumours from 0.5 to 2.3. Non-diploid T3 tumours had only one abnormal stem line (Fig. 2), while more than one abnormal stem line was seen among the T4 tumours (Fig. 2).

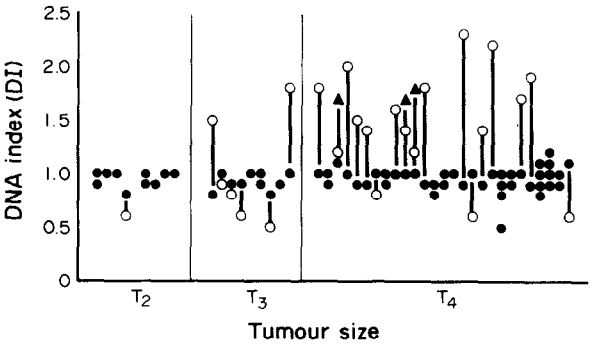

Fig. 2. Scattergram showing relationship between DI and tumour size among the oral tumours. Closed circles (●) show DI values of the G0/G1 peak of unimodal tumours and of the diploid stemline of the bimodal and multimodal tumours. Open circles (○) and (▲) drawn above the closed circles show DI value of the second and third stemline in the same tumour.

Table 3. Ploidy distribution and SpF among tumours of different grades

Grade (*n*)	Diploid	Non-diploid	SpF Mean ± S.D.
WD (18)	8 (44.4%)	10 (55.6%)	18.7 ± 6.3
MD (34)	24 (70.5%)	10 (29.4%)	18.8 ± 7.9
PD (11)	3 (27.8%)	8 (72.7%)	20.8

χ^2; $P<0.01$.

Table 4. Relation between ploidy, node involvement and tumour size

	Diploid		Non-diploid	
Size (*n*)	N+	N−	N+	N−
T2 (10)	3 (33.3%)	6 (66.6%)	0	1
T3 (10)	2 (50%)	2 (50%)	5 (66.7%)	2 (33.3%)
T4 (41)*	8 (42.1%)	11 (57.8%)	17 (77.2%)	5 (22.7%)

*CLNM status of one tumour was not known.

Ploidy distribution within different grades of the tumours is shown in Table 3. Among the well differentiated (WD) tumours, the diploid and non-diploid tumours were equally distributed. Moderately differentiated (MD) tumours were predominantly diploid and a majority of the poorly differentiated (PD) tumours were non-diploid. This difference in the distribution of ploidy in relation to the grade of the tumour was found to be statistically significant by χ^2 analysis ($P<0.01$). There was no correlation between the SpF and histopathological grade of the tumours.

34 of the 63 tumours (54%) (Table 1) had cervical lymph node metastasis. Nodal status of one tumour was not known. The non-diploid tumours showed a higher tendency for lymph node metastasis (69.2%) when compared to diploid tumours (42.8%) [χ^2; $P<0.05$].

Table 4 shows the relation between ploidy and nodal status among the T2, T3 and T4 tumours. Seven non-diploid tumours which were N−, were all well differentiated except one which was a poorly differentiated tumour.

A comparison of the SpF, carried out to distinguish between diploid, N+, T4 tumours from the diploid, N−, T4 tumours showed a significantly higher mean SpF in the former group (22.4 ± 4.1) than in the latter (16.9 ± 4.8) [*t*-test, mean $P<0.001$]. Such a comparison could not be made for the T3 tumours due to their small number.

The percentage of N+ tumours was highest among the poorly differentiated tumours and the percentage of N− tumours was highest in the well differentiated tumours. All the PD, N+ tumours, were non-diploid (Table 5). The MD, N− tumours were diploid except one, which was tetraploid (Table 5). 58% of MD, N+ tumours were also diploid. These diploid, N+, MD tumours had higher SpF (Mean 22.1 ± 8.9) as compared with the diploid, N−, ND tumours, which had a mean SpF of 16.0 ± 5.1 (*t*-test, $P<0.01$).

Table 5. Relation between grade, node involvement and ploidy status in oral tumours

	Diploid		Non-diploid	
Grade (*n*)	N+	N−	N+	N−
WD (18)	4 (44.4%)	5 (55.5%)	4 (44.4%)	5 (55.5%)
MD (34)	10 (41.6%)	14 (58.3%)	9 (90%)	1 (10%)
PD (8)	0	1	5 (71.4%)	2 (8.6%)

DISCUSSION

Squamous cell carcinoma of the oral cavity is a formidable problem in India. Several efforts have been directed at understanding the molecular basis of its aetiology. Although studies on alterations in cellular DNA content by flow cytometry are not directly aimed at understanding the genetic basis of the disease, several workers have used it as a marker in their efforts to find correlation with the course of the disease; as an aid in prognosis and in the management of the disease. Studies on carcinomas of the bladder, ovary, colorectum, cervix-uteri and breast, suggest that DNA analysis by flow cytometry is a useful prognostic tool along with histopathological and clinical features [4, 5].

The present study was aimed at identification of subsets amongst oral tumours with similar histopathological and clinical presentations, ultimately to achieve reliable prediction of tumour behaviour.

The tumours were grouped into diploid and non-diploid categories. There were more diploid tumours in this series, the percentage of aneuploid tumours being 43%. A correlation between tumour size and ploidy was observed. The T2 tumours were mainly diploid. Similar observations were made by Hemmer *et al.* [10] and Tytor *et al.* [11, 12]. The non-diploid T4 tumours were distinguished by two or more abnormal stem lines and high DI values (up to 2.3) from the non-diploid T3 tumours.

The proportion of non-diploid tumours was significantly higher among the poorly differentiated tumours. Such a pattern has also been reported in other studies [10–14].

Cervical lymph node involvement has always been an important clinical parameter influencing the prognosis of oral cavity carcinomas [15, 16]. The non-diploid tumours had a higher percentage of nodal metastasis than the diploid. This has also been reported by other workers [10–12, 17].

Borges *et al.* [9] have emphasised the need for pre-operative identification of the N− tumours among the T3, T4 SCC of buccal mucosa as clinical assessment of nodes is known to show false positives in 60% of these cases. Our results showed that N+, T4 tumours were usually non-diploid and the N−, T4 tumours were usually diploid. The diploid, N+, T4 tumours were distinguished by their higher SpF (>20%) from the diploid, N−, T4 tumours. This is supported by results of another study carried out by *in vitro* bromodeoxyuridine labelling of SCC of the oral cavity [18]. The labelling indices of N+ tumours were higher than those of the N− tumours. Though the present series consists of mixed tumours of the oral cavity, it seems possible to identify the N+ and N−, T4 tumours by flow cytometric measurements. Such a correlation between node involvement, ploidy and SpF was also observed among the moderately differentiated tumours. The diploid, N+, MD tumours had higher SpF than the diploid, N−, MD tumours.

Thus, in SCCs of the oral cavity, ploidy status correlates with tumour size, the poorly differentiated tumours are mainly

non-diploid and also N+ tumours are usually non-diploid. Amongst the T4 tumours and the moderately differentiated tumours it is possible to identify diploid, N+ tumours from diploid N− tumours by their higher S-phase fraction.

The results of this pilot study suggest that flow cytometric parameters may be useful biological correlates of tumour behaviour and may help understand different behaviour of similarly staged and graded tumours.

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