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DNA Content and PCNA Immunoreactivity in Oral Precancerous and Cancerous Lesions

Rüdiger G. Steinbeck, Jürgen Moege, Kerstin M. Heselmeyer, Werner Klebe, Wolfgang Neugebauer, Bent Borg and Gert U. Auer

Thirty-three dysplastic lesions showing varying degrees of atypia located in the oral cavity and 83 squamous cell carcinomas located in the oral cavity and tongue ($n=56$) or in the lips ($n=27$) were analysed by means of proliferating cell nuclear antigen (PCNA) immunoreactivity and image cytometric DNA measurements. The results show that in dysplastic lesions increasing cellular atypia correlated to elevated proliferative activity and aneuploidy occurring in the basal cell layers. In highly differentiated squamous carcinomas increased PCNA immunoreactivity and aneuploidy was preferentially observed focally (grade 1 tumours) or in the invasive zones (grade 2 tumours). In contrast, more poorly differentiated carcinomas (grade 3 and 4 tumours) showed strongly elevated proliferative activity and aneuploidy throughout the entire tumour mass.
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INTRODUCTION

INVASIVE SQUAMOUS carcinomas of the oral cavity represent a type of malignant tumour with a generally poor prognosis in spite of improved therapeutic procedures. In order to reduce the mortality-rate in oral cavity carcinomas the detection of early carcinomas or precancerous lesions is of utmost importance.

The histomorphological characterisation of dysplastic lesions and their discrimination from non-specific mucosal alterations has contributed to sharpen the detection of precancerous lesions. However, it is well-known that subjective morphological criteria are not always sufficient in order to identify premalignant cellular alterations.

It has been shown in both experimental animal models [1, 2] and clinical investigations that the development of squamous cell carcinomas, for example in the bronchial tree [3] and the uterine cervix [4] is paralleled by a progressive increase in nuclear DNA content resulting in a transition of diploid into aneuploid cell populations. It is suggested that this process reflects a replacement of genetically stable diploid cells by genetically unstable aneuploid cells. It has also been hypothesised that aneuploidy in preinvasive lesions still may be a reversible cellular alteration but nevertheless clearly indicates obligatory precancerous changes in the development of squamous cell carcinomas [5].

Another objective marker which has been frequently used in the evaluation of human tumours is the PC 10 antibody detecting PCNA (proliferating cell nuclear antigen). PCNA can be detected immunohistochemically, and is suggested to discriminate growth arrested cells (G_0) from proliferating cells (G_1, S, G_2). Using antibodies of type PC 10 it is possible to detect PCNA in formalin-fixed, paraffin-embedded archival specimens. A decisive advantage of image cytometric and immunohistochemical methods is the performance of DNA and PCNA analysis in morphologically identified tumour compartments or individual cells.

The aim of this study was to investigate whether biological markers such as DNA content analysis and PCNA immunoreactivity can be of help in the diagnosis of precancerous and cancerous lesions of the oral cavity, tongue and the lips.

MATERIALS AND METHODS

Samples

The material comprised 122 specimens from 117 patients. The histopathological diagnosis was as follows: six specimens were judged as normal mucosa with slight inflammation (mean patient age: 66.0 years), 33 specimens exhibited leukoplakia simplex or leukoplakia erosiva (mean patient age: 55.4 years), 56 specimens showed oral cavity or tongue carcinomas (mean patient age: 60.4 years) and 27 biopsies represented carcinomas of the lips (mean patient age: 64.2 years). The classification of the dysplasias was performed according to Burkhardt and Maerker [6], whereas the carcinomas were classified as described below. From each specimen adjacent histologic sections were prepared in the following way:

(1) One 4 μm section for haematoxylin and eosin staining for diagnostic histology.
(2) One 4 μm section for immunohistochemical PCNA analysis.
(3) Four 4 μm sections for Feulgen staining and DNA analysis.

Histological malignancy grading

A modification of the malignancy grading recommended by Anneroth and Hansen [7] was applied in each squamous cell

Correspondence to R.G. Steinbeck.
R.G. Steinbeck, J. Moege and K.M. Heselmeyer are at the Institute of Pathology, Stuhrsallee 29, D-2390 Flensburg, Germany; G.U. Auer is at the Department of Tumor Pathology, Karolinska Institute and Hospital, S-10401 Stockholm, Sweden; W. Klebe and W. Neugebauer are at Süderhofenden 12, D-2390 Flensburg, Germany; and B. Borg is at Norderstr. 52, D-2390 Flensburg, Germany.
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carcinoma case using four morphological parameters concerning the histomorphological pattern of invasion, the degree of keratinisation, the nuclear polymorphism and the number of mitoses. Each feature was scored from 1–4 points and the score of each variable was added into a total malignancy score for each tumour. The total malignancy score was grouped as follows: 1–4 = grade 1, 5–8 = grade 2, 9–12 = grade 3 and 13–16 = grade 4.

Immunohistochemistry

Staining procedure. The formalin-fixed paraffin-embedded tissue sections were mounted on glass slides coated with ordinary glue and oven-dried for 2 h at 55°C. The sections were deparaffinised in xylene and passed through a graded ethanol series. They were fixed in acetone for 5 min and incubated in 1% NP40 for 5 min. After thoroughly rinsing with water the sections were immersed for 30 min in 0.5% H_2O_2 prepared in distilled water to block endogenous peroxidase activity. Sections were subsequently washed in water and TPBS (Tris and phosphate buffered saline) and then immersed for 45 min in 1% BSA (bovine serum albumin) prepared in TPBS to reduce non-specific antibody binding. PC 10 antibody (Novocastra, Newcastle upon Tyne, U.K.) at a dilution of 1:75 in 1% BSA (in TPBS) was applied and the sections were incubated overnight coverslipped in a humid chamber. After washing with TPBS (three changes of 5 min each) biotinylated anti-mouse IgG (Vector, Burlingame, U.S.A.), diluted 1:200 in TPBS, was applied for 30 min. The sections were subsequently washed in TPBS (three changes of 5 min each). The ABC solution (Vectastain, Elite ABC Kit, Vector, Burlingame, U.S.A.) was prepared according to manufacturer's recommendations and applied for 45 min. After washing in TPBS (three changes of 5 min each) diaminobenzidine-hydrogen peroxide (Sigma) was used as a chromogen. Finally a light Mayer's haematoxylin counterstain was applied and the sections were dehydrated in alcohol, cleared in xylene, and mounted in Pertex (Histolab, Göteborg, Sweden).

Evaluation of PCNA immunoreactivity. Only cells with a distinct brown staining confined to the nuclei were regarded as PCNA positive. Histological sections from lymph nodes were used as external positive (central part) and negative (peripheral part) controls. PCNA immunoreactive cells were counted in morphologically selected areas of each lesion. In normal and dysplastic mucosa counting was done separately in the 3–4 cell layers closest to the basal membrane and in the corresponding superficial layers including the great intermediate cells. In grade 2 squamous carcinomas counting was performed separately in the 2–3 most peripheral cell layers of the tumour islets and of the mature central part. In grade 3 and 4 squamous carcinomas the percentage of PCNA positive cells were calculated in the entire growing zone. Each calculation was based on at least 200–300 cell counts.

DNA analysis

Tissue preparation. Approximately 4 μm thick sections were cut from formalin-fixed, paraffin-embedded, histopathological specimens. The sections were deparaffinised and refixed in formalin.

Staining procedure. The tissue preparations were rehydrated in decreasing ethanol concentrations and exposed to acid hydrolysis in 5 N HCl at 22°C for 60 min [8]. The specimens were then rinsed in distilled water and stained with Schiff's reagent for 90 min at room temperature. After rinsing in distilled water again, the samples were washed three times in sulphide rinsing solution (10 ml $Na_2S_2O_5$, 10 ml HCl, 180 ml distilled water). Finally they were rinsed under running tap water, dehydrated in an increasing ethanol scale, transferred to xylene, and mounted in Eukitt (refractory index 1.494).

Measuring procedure. The area for DNA measurement was selected and marked by morphological criteria. Within the selected areas DNA measurement in individual cell nuclei was performed at random.

The specimens were evaluated on a TV-based image analysis system (AHRENS System, Bargteheide/Hamburg, F.R.G.), using a microscope (Nikon, plan objective 40/0.95) equipped with a video-CCD camera. In each case at least 100 structurally identified neoplastic cell nuclei were measured, avoiding nuclei with indistinct nuclear membranes, and nuclei lying too close together, or overlapping each other. As an internal standard, at least 20 granulocytes or lymphocytes were assessed, and their median value was accepted as the "diploid" or "2c" DNA value.

Interpretation of histograms. The DNA histograms obtained by image cytometry were subdivided into four types [9]. Type I ("diploid") shows a single peak in the "diploid" or "near-diploid" region of normal cells. Type II ("diploid-tetraploid") is characterised by a single peak in the "tetraploid region" or a peak in both the "diploid" and "tetraploid" region (>20% of the total cell population). The total amount of cells with DNA-values between the "diploid" and "tetraploid" region and those exceeding the "tetraploid" region is <5%. Type III shows a distribution pattern comparable with that found in proliferating normal populations, i.e. a main peak in the "diploid" region, a reasonable number of cells in the S-phase region (>5%) and a minor peak in the "tetraploid" region (<20%). Type IV is characterised by increased and/or scattered DNA-values significantly exceeding the "tetraploid" region.

RESULTS

Immunohistochemical PCNA and DNA content analyses were performed separately in the basal and superficial cell layers (c.f. Material and Methods) of slightly inflammatory and dysplastic mucosa. Figure 1 shows that the percentage of PCNA immunoreactive cells as well as the DNA content increased progressively in the basal layers with an increasing grade of dysplasia. On the other hand except for the severe dysplasia cases, the superficial cell layers were characterised by no or low numbers of PCNA immunoreactive cells and cells with diploid or near-diploid DNA content.

Table 1 illustrates the relationship between histomorphological grading (c.f. Material and Methods) of squamous cell carcinomas located in either the oral cavity and tongue or the lips and DNA ploidy. It can be seen from the Table that increasing malignancy grade was correlated with increasing percentages of aneuploid carcinomas. It is also evident that the tumours located in the lips generally show higher grades of differentiation and lower numbers of aneuploidy as compared with tumours of the oral cavity or tongue.

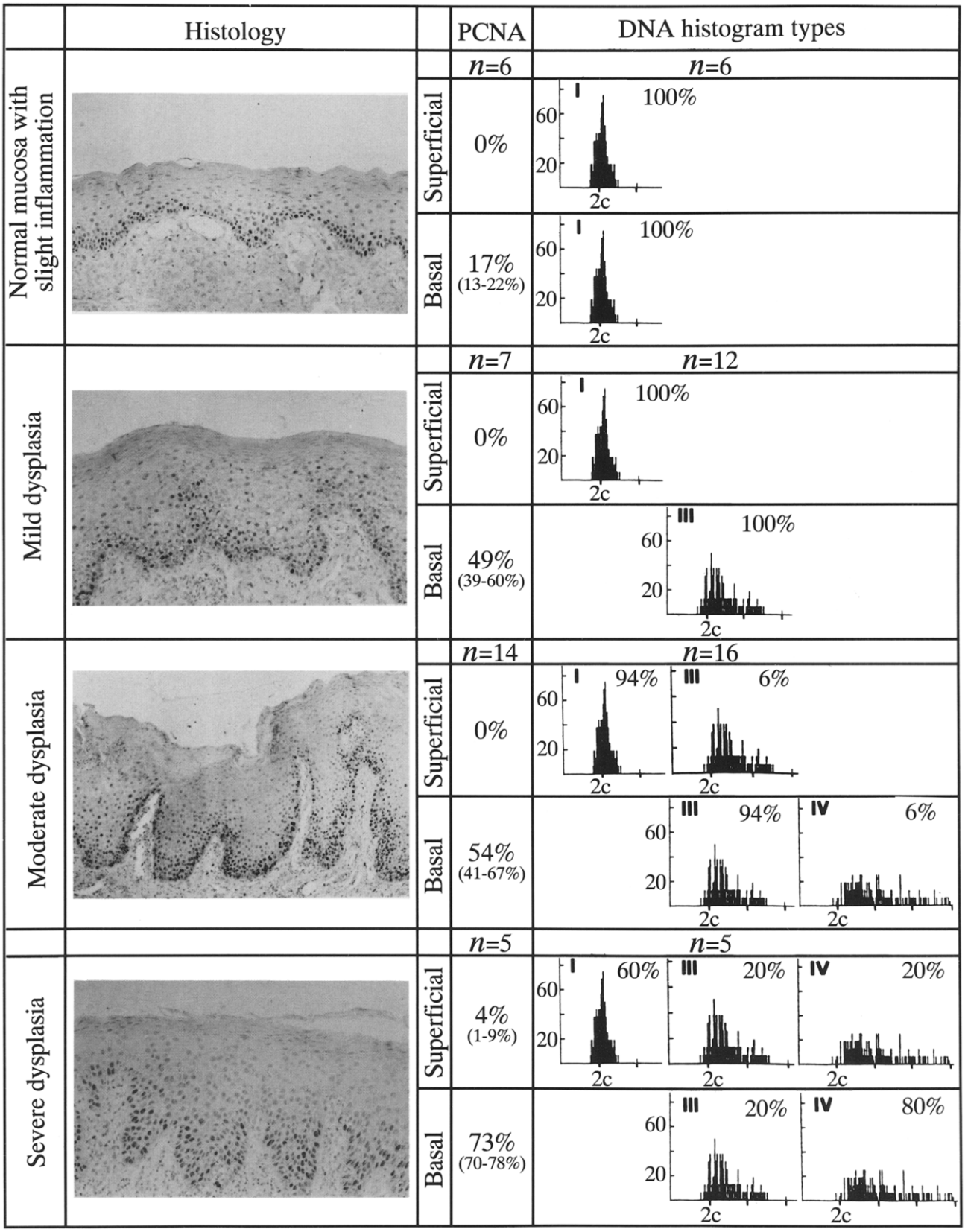

Fig. 1. Proliferative activity (PCNA immunoreactivity) and DNA distribution measured separately in the basal and superficial layers of normal oral mucosa and oral mucosa with mild, moderate and severe atypia (dark nuclei = PCNA immunoreactive nuclei).

Table 1. DNA ploidy measured in histomorphologically graded squamous cell carcinomas of the oral cavity and tongue (n=56) *and of the lips* (n=27)

	Number of cases (%)	DNA ploidy		
		Type I	Type III	Type IV
Oral cavity/tongue				
Grade 1	2 (3.6%)	—	—	2 (100.0%)*
Grade 2	15 (26.8%)	—	2 (13.3%)†	13 (86.7%)†
Grade 3	13 (28.6%)	—	2 (12.5%)	14 (87.5%)
Grade 4	19 (41.0%)	—	1 (4.3%)	22 (95.7%)
Total	56 (100.0%)	—	5 (8.9%)	51 (91.1%)
Lips				
Grade 1	6 (22.2%)	2 (33.3%)	2 (33.3%)	2 (33.3%)
Grade 2	11 (40.8%)	—	6 (54.5%)	5 (45.5%)
Grade 3	9 (33.3%)	—	—	9 (100.0%)
Grade 4	1 (3.7%)	—	—	1 (100.0%)
Total	27 (100.0%)	2 (7.4%)	8 (29.6%)	17 (63.0%)

*Measurements were done in morphologically deviating small areas.
†Measurements were done in the invasive peripheral zones of the tumour islets.

Figure 2 illustrates PCNA immunoreactivity (dark nuclei) in squamous carcinomas showing characteristic histopathological features and growth patterns of grade 1–4 tumours (c.f. Materials and Methods). It is clear from the photographs that PCNA immunoreactivity increases with increasing grade of malignancy.

Tumours morphologically judged as grade 2 squamous cell carcinomas with pronounced central cornification exhibited a heterogeneous PCNA distribution pattern and differing DNA profiles. DNA measurements in this subtype showed that aneuploid DNA distribution patterns were found in as many as 90% of the peripheral regions whereas all cells from the

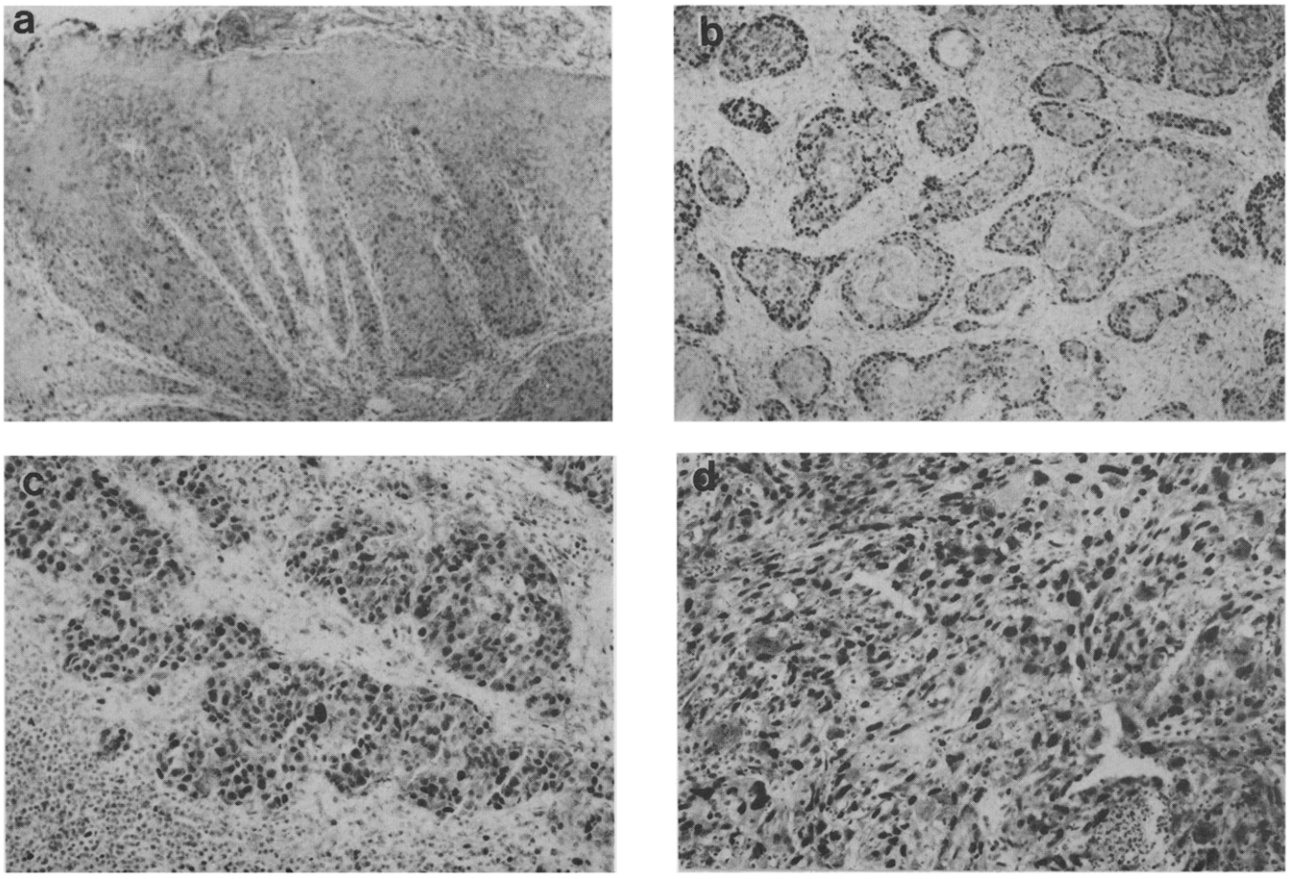

Fig. 2. Proliferative activity (PCNA immunoreactivity; dark nuclei=PCNA immunoreactive nuclei) in squamous cell carcinomas showing characteristic histopathological features and growth patterns of grade 1–4 tumours (a=grade 1; b=grade 2; c=grade 3; d=grade 4).

central cornified parts of the tumour islets had diploid or near-diploid DNA profiles (Fig. 3). PCNA immunoreactivity was more frequent in the peripheral cell populations and nearly absent in the central cornified parts of the tumour. In the two grade 1 tumours included in this study small areas composed of aneuploid cells could be observed in the peripheral zones, whereas the main tumour population was diploid.

The lower differentiated (grade 3 and 4) tumours showed a scattered, in general significantly elevated number of PCNA immunoreactive cells with tumour areas exhibiting between 57 and 87% (mean = 79%) proliferating cells.

DISCUSSION

The results reported herein support the hypothesis that squamous cell carcinomas of the oral cavity develop through a sequence of cellular alterations in part reflected by increasing nuclear proliferative activity and DNA content. It was also found that these markers generally correlated with the histomorphological classification as proposed by Anneroth and Hansen [7]. Thus, major areas of the highly differentiated grade 1 tumours and the differentiated cornified central areas of the grade 2 squamous cell carcinomas exhibited low numbers of PCNA immunoreactive cells and diploidy whereas the peripheral zones of grade 2 tumours as well as the entire tumour populations of grade 3 and 4 squamous cell carcinomas were dominated by high frequencies of PCNA positive cells and aneuploidy.

However, even in grade 1 tumours small areas, usually located in the peripheral zones of a tumour islet, can exhibit elevated PCNA immunoreactivity and aneuploidy. It may be suggested that the expansion of these aneuploid populations can explain the transition of some grade 1 tumours into more poorly differentiated tumours.

The coexistence of tumour regions in grade 2 tumours dominated by aneuploid or diploid cells is interesting since it may indicate the possibility that highly aneuploid cells under certain circumstances may differentiate into diploid cells. The mechanism behind this biological behaviour is unknown. One hypothetical explanation could be that the pronounced mitotic failure observed in aneuploid cell populations resulting in a large variety of daughter cells with differing karyotypes may produce near-diploid cells with the property to differentiate.

The demonstration of small aneuploid populations in highly differentiated tumour types is dependent on the method of quantitative DNA analysis. Using flow cytometry these small populations are hardly detectable resulting in the fact that such a tumour will be reported as entirely diploid. In contrast, DNA analysis in histological sections makes it possible to measure even small cell populations which can be histomorphologically or immunohistochemically identified. A drawback of DNA measurements in histological sections is the fact that nuclei may be cut resulting in uneven DNA profiles and increased coefficients of variation of the stemline(s). However, methodological studies showed that DNA measurements can be performed in sectioned material with relatively high accuracy. Thus, no major difference in the number of diploid or aneuploid cases was found when comparing DNA histograms of imprints and sections from the same specimens [10].

The high percentage of aneuploid tumours is in line with the well-known poor prognosis of invasive squamous carcinomas of the oral cavity and tongue. In this context it is of interest to notice that squamous invasive carcinomas of the lips which in general have a better prognosis also exhibited lower percentages of aneuploid tumours in our material.

Due to a short follow-up of the majority of cases it was not the aim of this study to evaluate in a multivariate analysis the possible additional prognostic information obtainable by nuclear DNA content or PCNA analysis. It is therefore at present not possible to estimate whether the biological markers used herein add prognostic information over and above that obtained by histopathological classification. However, it

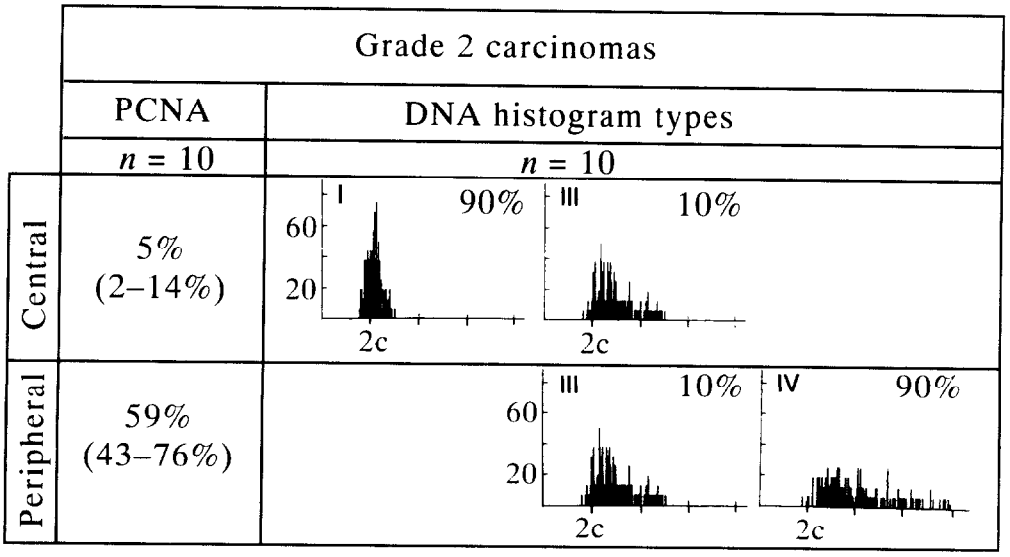

Fig. 3. Proliferative activity (PCNA immunoreactivity) and DNA distribution in grade 2 carcinomas of the oral cavity. The analyses were performed separately in the cornified central parts and the invasive peripheral zones.

appears that markers analysing the degree of genetic instability and proliferative activity, e.g. DNA and total PCNA can be of importance for the early detection of precancerous lesions in the oral cavity, tongue and lips.

An important observation of this study is that the malignancy-related cellular alterations seen in the dysplastic lesions of the oral cavity generally occur in the basal cell layers and might not be present in the superficial cell layers. This indicates that cytological smears or flat surgical biopsies might not result in representative diagnostic material.

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