DNA CONTENT AS A PROGNOSTIC MARKER IN PATIENTS WITH ORAL LEUKOPLAKIA

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ABSTRACT

Background Oral leukoplakia may develop into squamous-cell carcinoma, which has a poor prognosis. Risk factors for oral carcinoma have been identified, but there are no reliable predictors of the outcome in individual patients with oral leukoplakia.

Methods We identified 150 patients with oral leukoplakia that was classified as epithelial dysplasia and measured the nuclear DNA content (ploidy) of the lesions to determine whether DNA ploidy could be used to predict the clinical outcome. Biopsy specimens obtained at annual follow-up visits were graded histologically and classified with respect to DNA content in a blinded fashion. Disease-free survival was assessed in relation to DNA ploidy and the histologic grade. The mean duration of follow-up was 103 months (range, 4 to 165).

Results Among 150 patients with verified epithelial dysplasia, a carcinoma developed in 36 (24 percent). Of the 150 patients, 105 (70 percent) had diploid (normal) lesions, 20 (13 percent) had tetraploid (intermediate) lesions, and 25 (17 percent) had aneuploid (abnormal) lesions at the time of the initial diagnosis. A carcinoma developed in 3 of the 105 patients with diploid lesions (3 percent), as compared with 21 of the 25 patients with aneuploid lesions (84 percent), yielding a negative predictive value of 97 percent with respect to the diploid lesions and a positive predictive value of 84 percent with respect to the aneuploid lesions. Carcinoma developed in 12 of 20 patients with tetraploid lesions (60 percent). The mean time from the initial assessment of the DNA content to the development of a carcinoma was 35 months (range, 4 to 57) in the group with aneuploid lesions and 49 months (range, 8 to 78) in the group with tetraploid lesions ($P=0.02$). The cumulative disease-free survival rate was 97 percent among the group with diploid lesions, 40 percent among the group with tetraploid lesions, and 16 percent among the group with aneuploid lesions ($P<0.001$).

Conclusions The DNA content in cells of oral leukoplakia can be used to predict the risk of oral carcinoma. (N Engl J Med 2001;344:1270-8.)

WHITE patches (leukoplakia) of the oral cavity have a well-documented potential to develop into squamous-cell carcinoma,[1-3] and when this occurs, the odds of surviving more than five years are poor.[4,5] Accurate prognosis is important in patients with oral leukoplakia, because with avoidance of the use of tobacco and alcohol and appropriate treatment, malignant disease can be averted.[6,7]

Between 5 and 15 percent of oral white patches are classified histologically as dysplasia.[8,9] Of these, 15 to 20 percent develop into carcinoma.[10] Since histologic assessment of these dysplastic lesions is of limited prognostic value,[11] therapeutic intervention is considered only in cases in which there is histologic evidence of the transition to carcinoma in situ or carcinoma. Molecular markers have been investigated, but they lack prognostic value in precancerous oral lesions.[12-17]

Compelling evidence points to abnormalities in the number of chromosomes (aneuploidy) as a cause rather than a consequence of malignant transformation.[18] Moreover, mutations in genes controlling the segregation of chromosomes during mitosis and abnormalities of the centrosome are critical in the chromosomal instability of cancer.[19-23] Chromosomal aberrations consistent with the occurrence of abnormal chromosomal segregation during mitosis occur exclusively in aneuploid tumor-cell lines.[24] All these observations point to the importance of an aberration of DNA content (ploidy) in carcinogenesis.[25]

Three previous studies of the prognostic value of DNA quantitation in premalignant oral lesions included fewer than 25 patients with at least five years of follow-up.[26-28] In this report we describe the prognostic value of the DNA content in dysplastic oral leukoplakia from 150 patients who were repeatedly observed for a mean of almost nine years.

METHODS

Ascertainment of Dysplasia

Between 1982 and 1995, excisional-biopsy specimens were obtained from 242 patients with oral white patches at three institutions in Norway (University Hospital in Bergen and the Department of Otolaryngology and the Department of Oral Surgery and Medicine at the University of Oslo in Oslo). All histologic sections were subsequently reevaluated by four pathologists according to the guidelines of the World Health Organization.[9] Consensus on the classification of dysplasia was reached in the case of 196 of the 242 patients (81 percent). Of these 196 patients, 36 had already been given a diagnosis of carcinoma in situ or carcinoma of the oral cavity and were therefore not included (Fig. 1). Of the 160 remaining

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patients, ample tissue blocks were available for 150. Hence, 150 patients with white patches that were classified as dysplastic were included in the study. All sections were coded, and the histologic typing and grading were done in a blinded fashion.

All 150 patients had been reported to the Cancer Registry of Norway and enrolled in a follow-up program, which, through an updated national register, had hospital-based access to the place of residency of Norwegian citizens. No upper limit was set for the duration of follow-up. Patients who were given a diagnosis of dysplasia were scheduled to have an annual examination, which included inspection of the oropharyngeal mucosa and palpation of cervical lymph nodes. Biopsies were performed at these follow-up visits if previously unrecognized white patches were detected, white patches recurred after excision, or previously recognized patches had increased in size. No patients were lost during follow-up, although data on seven patients who died of unrelated causes were censored at the time of death.

Complete excision of visible white patches was attempted in the case of all single lesions that were 3 cm in diameter or less. In the case of multiple lesions or lesions that were larger than 3 cm in di-

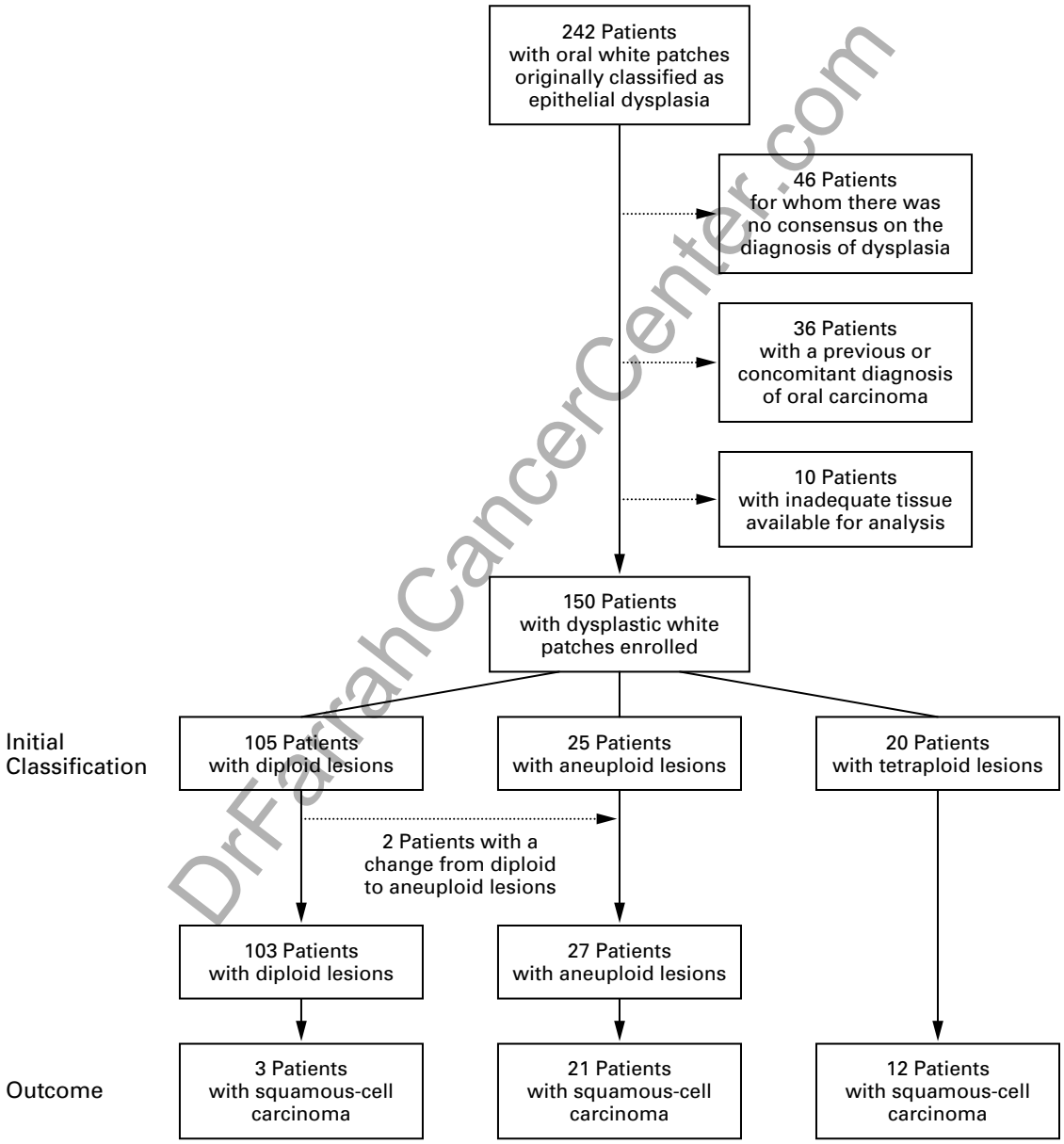

Figure 1. Selection, Classification, and Outcome of Patients with Oral Leukoplakia According to the Analysis of the DNA Content of the Lesions.

There were no changes in ploidy from aneuploid to either tetraploid or diploid or from tetraploid to either diploid or aneuploid. Carcinoma did not develop during follow-up in either of the patients with lesions that changed from diploid to aneuploid during follow-up.

ameter, further excision was done in patients with histologically verified dysplasia. The locations of the lesions and subsequent carcinomas were documented with the use of the topographic codes in the *Systematized Nomenclature of Medicine*.[29] Only carcinomas that developed in the same location as or in the vicinity of previous white patches were regarded as instances of disease progression.

Patients with confirmed use of tobacco or alcohol were given standard oral and written information on risk factors for oral cancer, and this information was repeated at each follow-up visit. Data on tobacco use were reconstructed from the medical records or by the use of telephone interviews, in which the patients were asked about their use of tobacco at the time of the initial diagnosis of oral leukoplakia (no history of tobacco use, former use of tobacco, or use of tobacco at the time of the initial diagnosis).

Staining of Tissue Samples

Paraffin-embedded tissue samples fixed in 4 percent buffered formaldehyde were sectioned (Fig. 2). To increase the sensitivity of the analysis, the pathologists identified the dysplastic areas and removed the parts of the blocks peripheral to the lesions. Two 50-μm sections were cut and enzymatically digested (type XXIV protease, Sigma Chemical, St. Louis) to yield isolated nuclei and subsequently a monolayer.[30] Adjacent sections stained with hematoxylin and eosin were analyzed in order to verify the dysplastic content of each tissue sample.

Measurement of DNA Content

The DNA content of nuclei stained with Feulgen's stain and periodic acid–Schiff stain was measured and analyzed with use of the Fairfield ploidy system (Fairfield Imaging, Kent, United Kingdom), according to an established protocol (Fig. 2).[31] Monolayers were analyzed with use of a Zeiss Axioplan II microscope (Zeiss, Oberkochen, Germany) (40× lens, or eyepiece, and 0.65 objective) that was equipped with a 546-nm green filter and that had been modified so that the staging could be controlled by a computer model (HI52V2, Prior Scientific Instruments, Fulbourn, Cambridge, United Kingdom). The microscope was also equipped with a single-chip digital camera (model C4742-95, Hamamatsu Photonics, Hamamatsu, Japan). The final magnification was ×1600 at an estimated resolution of 170 nm (0.2 μm) per pixel; the visual field measured 1024 by 1024 pixels and had a 10-bit resolution (1024 gray levels). The nuclei of at least 300 cells were measured, and the information was stored in a computerized folder, or "gallery," for each patient, and lymphocytes were included as internal controls. The DNA content was measured in biopsy specimens obtained at the time of the initial diagnosis of dysplasia and at follow-up visits. The mean coefficient of variation of the DNA content in nuclei during the diploid peak, as registered by the number of nuclei in which there are two sets, or two copies (2c), of each chromosome, was 5.7 percent (range, 3.3 to 7.9) for all 150 patients.

Criteria for the Classification of DNA Content

All specimens were coded, and DNA histograms were classified in a blinded manner by four observers. In patients from whom multiple biopsy specimens were obtained simultaneously, all specimens were analyzed for the DNA content and the most abnormal DNA classification was chosen if the results were discrepant. A lesion was classified as diploid if there was only one peak (which was 2c) during the G_0 or G_1 phase, if the number of 4c nuclei during the peak of the G_2 phase did not exceed 10 percent of the total, or if the number of nuclei with a DNA content of more than 5c did not exceed 1 percent of the total. A lesion was defined as tetraploid when there was a 4c peak during the G_0 or G_1 phase together with an 8c peak during the G_2 phase or when the number of 4c nuclei during the peak of the G_2 phase exceeded 10 percent of the total. A lesion was defined as aneuploid if there were aneuploid peaks (3c, 5c, 7c, or 9c) or if the number of nuclei with a DNA content of more than 5c or 9c exceeded 1 percent of the total. Examples of biopsy specimens with corresponding DNA histograms are shown in Figure 3.

Statistical Analysis

Survival curves as they related to the DNA content and the histologic grade of severity were constructed according to the Kaplan–Meier method. The end point was the development of oral squamous-cell carcinoma. Data on a patient were censored in the Kaplan–Meier estimate if the patient died of an unrelated disease. The log-rank test was used to assess the prognostic value of the DNA content in relation to disease-free survival. Differences in proportions were evaluated with use of the chi-square test. Bivariate correlation analysis was used to assess the correlation between the severity of dysplasia and the DNA content. A Cox proportional-hazards regression model was used for the multivariate analysis. The size of the lesions and the presence or absence of alcohol consumption were not included in the multivariate analysis, since reliable data on these variables were not available. The cumulative risk of a carcinoma was calculated in the form of odds ratios, which were adjusted for age, sex, and tobacco-use status and reported with the corresponding 95 percent confidence intervals. All P values were two-sided, and P values of less than 0.05 were considered to indicate statistical significance. SPSS statistical software (SPSS, Chicago) was used for the calculations.

RESULTS

Characteristics of the Patients

Table 1 summarizes the main characteristics of the patients, and Table 2 shows the follow-up data, including the distribution of the 299 biopsy specimens evaluated. All biopsy specimens were evaluated by four independent observers and classified with respect to DNA content. The mean duration of follow-up after the initial diagnosis of dysplasia was 103 months (range, 4 to 165).

In 36 patients — 3 with diploid lesions, 21 with aneuploid lesions, and 12 with tetraploid lesions (24 percent) — an oral squamous-cell carcinoma developed on or near the sites of previous white patches, after a mean follow-up of 49 months (range, 4 to 78). In all but 1 of these 36 patients (a patient with diploid lesions), the carcinoma developed within five years after the initial diagnosis of dysplasia (Fig. 4). In the case of four patients two biopsy specimens apiece were obtained initially; since these patients had lesions that exceeded 3 cm in diameter, the lesions could not be completely excised in a single procedure, and additional procedures were needed because dysplasia was found in the first biopsy specimen. In all four patients, both biopsy specimens were classified as having the same DNA content; two had aneuploid lesions, and two had diploid lesions. In the two patients with aneuploid lesions, carcinoma developed after 38 and 50 months of follow-up. No patient had more than one biopsy at subsequent visits.

Of the 36 patients in whom carcinoma developed, successive biopsies were performed in 28 (78 percent). These specimens were obtained from various locations, most often along the lateral border of the tongue or the floor of the mouth, and were bilateral in the case of 16 patients. Data on seven patients were censored at the time of their death from other causes (five had diploid lesions, one had tetraploid lesions, and one had aneuploid lesions according to the classification of the

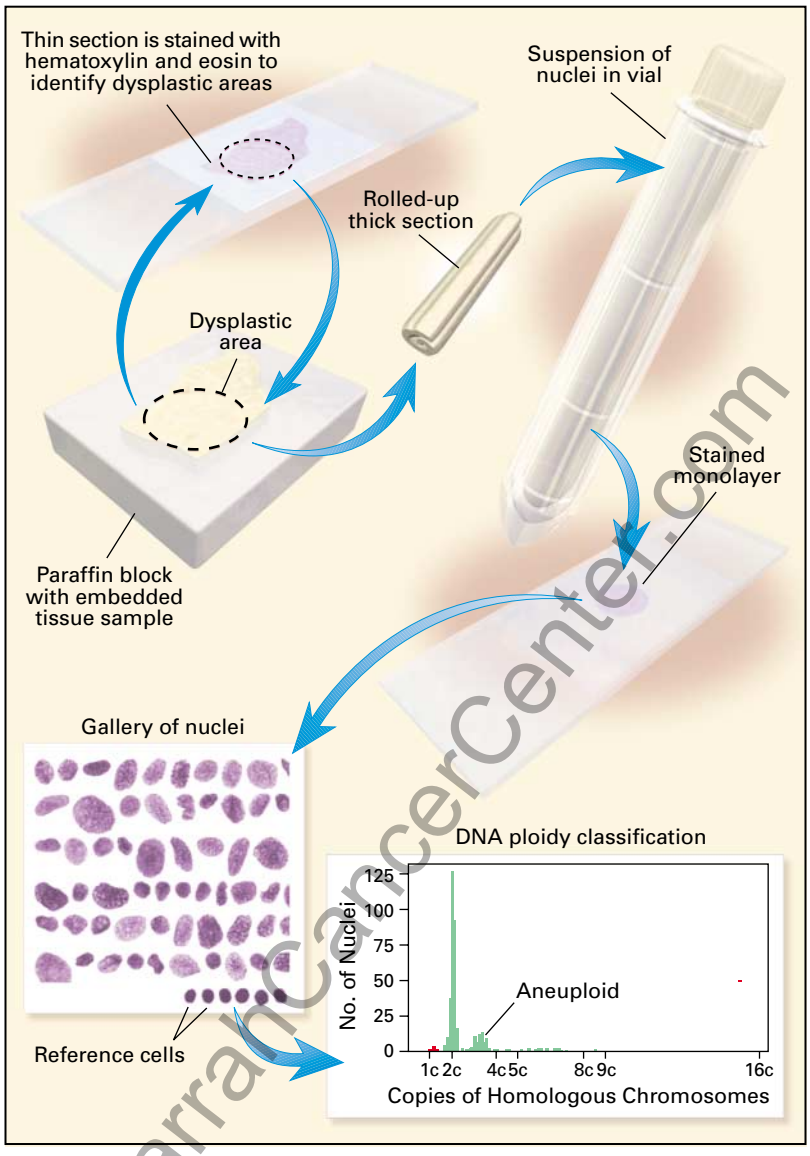

Figure 2. Steps in the Preparation of a Monolayer.

First, paraffin-embedded blocks of biopsy specimens are sectioned and stained with hematoxylin and eosin. The stained sections identify the dysplastic areas, and the uninvolved areas are trimmed off. Two sections with a thickness of 50 μm — one of which is shown rolled up here — are deparaffinized and rehydrated before being enzymatically digested to yield a suspension of nuclei. The suspension is then centrifuged, and the pellet is resuspended and then placed on a slide to form a monolayer. After staining with Feulgen's stain and periodic acid–Schiff stain, the nuclei are viewed under a microscope and the images are collected in a computerized folder, or "gallery," for each patient. At least 300 nuclei of dysplastic epithelial cells are analyzed together with nuclei from reference cells (lymphocytes). The slides with cell nuclei are then viewed with a transmission light microscope equipped with a digital camera. The amount of light transmitted is registered digitally by the camera and stored in the computer; it is inversely proportional to the amount of DNA in the nuclei. The final classification of DNA ploidy is made from the DNA histogram that is generated from the information in the gallery. In the histogram, the results for the reference-cell nuclei are shown in red and the results for the epithelial cells are shown in green. The y axis shows the number of epithelial-cell nuclei, and the x axis shows the nuclear DNA content according to the number of copies (c) of homologous chromosomes. The diploid (2c) peak (in green) is the first column to the right of the reference cells (in red).

initial biopsy specimens). No other patients were lost to follow-up.

DNA Ploidy

Of 105 patients who were initially classified as having diploid lesions (Table 1), 103 continued to have diploid lesions throughout the follow-up period (mean, 74 months; range, 37 to 103) and 2 had a change in ploidy to aneuploid lesions. Among these 105 patients, carcinoma developed in 3 after 35, 46, and 76 months. Only lesions classified as diploid at base line and in all follow-up observations were regarded as diploid in the analysis of survival (Fig. 1).

Among the 20 patients with tetraploid lesions (Table 1), no change in ploidy occurred during follow-up; a carcinoma developed in 12 of these patients (60 percent) after a mean of 49 months (range, 8 to 78). Of the 27 patients who were eventually classified as having aneuploid lesions, 25 had consistently aneuploid lesions (Table 1) and 2 had diploid lesions that became aneuploid during the follow-up period. Carcinoma did not develop in either of the two patients with a change in ploidy. Of the 25 patients with aneuploid lesions at base line, a carcinoma developed in 21 (84 percent) after a mean of 35 months (range, 4 to 57). There was a significant difference between the patients with tetraploid lesions and those with aneuploid lesions with respect to the time from the initial diagnosis to the development of carcinoma ($P=0.02$).

The cumulative disease-free survival rate was 97 percent in the group with diploid lesions, 40 percent in the group with tetraploid lesions, and 16 percent in the group with aneuploid lesions ($P<0.001$ by the log-rank test) (Fig. 4A). The cumulative relative risks of a carcinoma in the group with tetraploid lesions and in the group with aneuploid lesions, as compared with the group with diploid lesions, given as odds ratios, were 20.2 (95 percent confidence interval, 10.9 to 29.5) and 27.6 (95 percent confidence interval, 18.4 to 36.8), respectively.

In a subanalysis of subsequent ploidy data for all 150 patients, performed after the change in status from diploid to aneuploid in the lesions from 2 patients (after 43 and 62 months), the cumulative disease-free survival rate was 95 percent in the group with diploid lesions, 40 percent in the group with tetraploid lesions, and 18 percent in the group with aneuploid lesions ($P<0.001$), and the relative risks of oral carcinoma were 20.0 (95 percent confidence interval, 10.7 to 29.3) in the group with tetraploid lesions and 27.2 (95 percent confidence interval, 18.2 to 36.2) in the group with aneuploid lesions. The negative predictive value of the finding of diploidy in the initial biopsy specimen with respect to the subsequent development of a carcinoma was 97 percent, and the positive predictive value of an initial finding of aneuploidy was 84 percent.

Histologic Grades

Of the initial 150 biopsy specimens, 49 (33 percent) were histologically graded as showing mild dysplasia, 57 (38 percent) as showing moderate dysplasia, and 44 (29 percent) as showing severe dysplasia. The cumulative disease-free survival rate was 68 percent in the group with mild dysplasia, 78 percent in the group with moderate dysplasia, and 82 percent in the group with severe dysplasia ($P=0.33$) (Fig. 4B). There was no statistically significant correlation between the histologic grade, as judged by the four observers, and the DNA content.

Risk Factors for Oral Carcinoma

Information on tobacco use was obtained from the medical records of 100 patients. In addition, 37 patients could be reached by telephone and had clear recollections of their tobacco use (no history of the use of tobacco in any form, former use, or current use) at the time of initial diagnosis and reexaminations. Of these 137 patients, 27 (21 women) had never used tobacco. A carcinoma developed during follow-up in 5 of the 27 patients with no history of tobacco use and in 22 of the 85 patients who were current users of tobacco. Among the 13 patients whose use of tobacco was unknown, oral carcinoma developed in 2 during follow-up.

In univariate analysis, aneuploidy ($P<0.001$), current tobacco use ($P=0.03$), and poor dental hygiene (defined as severe untreated carious lesions, periodontitis, unsatisfactory dental restorations, and decubital lesions from poorly fitting removable dentures) ($P=0.05$) were significant prognostic factors, whereas age and sex were not ($P=0.39$ and $P=0.28$, respectively). When aneuploidy, current tobacco use, and poor dental hygiene were fitted in a multiple-regression model, aneuploidy and current tobacco use remained significant prognostic factors ($P<0.001$ and $P=0.05$, respectively).

DISCUSSION

Our results support the practice of watchful waiting with respect to patients who have oral leukoplakia with a normal (diploid) DNA content. By contrast, lesions with an abnormal (aneuploid) DNA content should be treated as true carcinomas. We found that the rate of malignant transformation of oral leukoplakia was substantial (24 percent), even though we excluded

Figure 3 (facing page). Ploidy (Insets) and Histologic Findings in Two Patients with Moderate Dysplasia (Panels A and B) and One Patient with Mild Dysplasia (Panel C) (Hematoxylin and Eosin). In each histogram, c denotes copy or copies, and the red columns to the left of the 2c (diploid) peak are internal controls. In the histogram shown in the inset in Panel B, "S phase to 4c" indicates cells in synthesis phase that are about to double their DNA content.

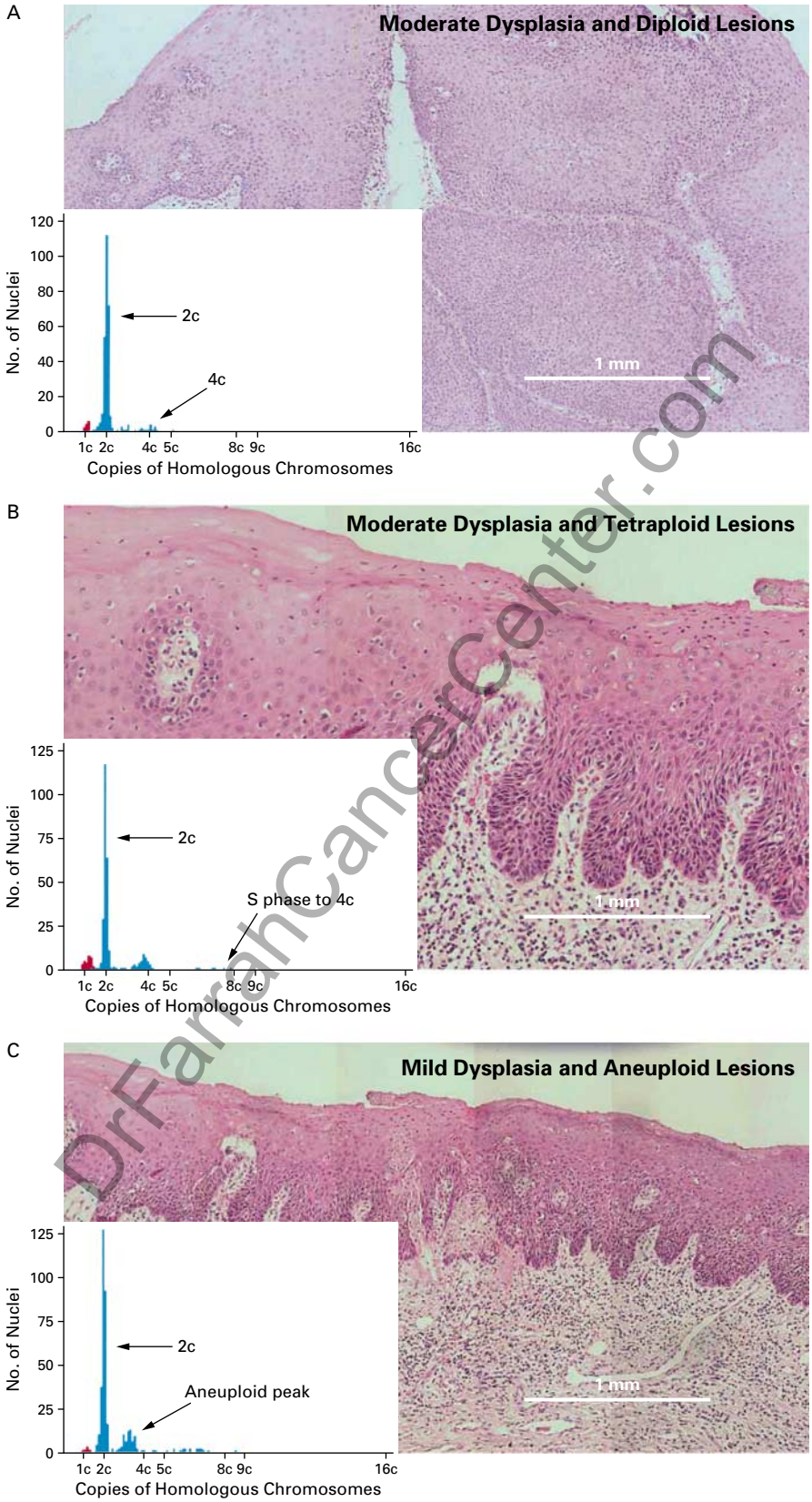

TABLE 1. CHARACTERISTICS OF THE 150 STUDY PATIENTS, ACCORDING TO THE INITIAL CLASSIFICATION OF THEIR ORAL LEUKOPLAKIA.*

CHARACTERISTIC	PATIENTS WITH DIPLOID LESIONS (N=105)	PATIENTS WITH TETRAPLOID LESIONS (N=20)	PATIENTS WITH ANEUPLOID LESIONS (N=25)
Mean age — yr	69.6	64.5	69.3
Male sex — no. (%)	59 (56)	10 (50)	12 (48)
Histologic grade of dysplasia — no. (%)			
Mild	35 (33)	3 (15)	11 (44)
Moderate	35 (33)	13 (65)	9 (36)
Severe	35 (33)	4 (20)	5 (20)
Tobacco use — no. (%)			
No history of use	21 (20)	3 (15)	3 (12)
Former use	17 (16)	3 (15)	5 (20)
Current use	62 (59)	10 (50)	13 (52)
Cigarettes	40 (38)	6 (30)	10 (40)
Smokeless tobacco	22 (21)	4 (20)	3 (12)
No information available	5 (5)	4 (20)	4 (16)
Current tobacco users with carcinoma	2 (2)†	3 (4)†	17 (20)†
Patients with no history of tobacco use with carcinoma	0‡	2 (7)‡	3 (11)‡
Poor dental hygiene — no. (%)§			
Yes	54 (51)	8 (40)	12 (48)
No	44 (42)	8 (40)	10 (40)
No information available	7 (7)	4 (20)	3 (12)
Patients with poor dental hygiene with carcinoma	3 (4)¶	4 (5)¶	3 (4)¶
Patients with good dental hygiene with carcinoma	1 (2)‖	3 (5)‖	2 (3)‖

*Because of rounding, percentages may not total 100.

†The percentages of patients in whom an oral carcinoma developed during follow-up are based on the 85 patients in the total study population who were using tobacco at the time of the initial diagnosis.

‡The percentages of patients in whom an oral carcinoma developed during follow-up are based on the 27 patients in the total study population who had never used tobacco.

§Poor dental hygiene was defined by the presence of severe untreated carious lesions, periodontitis, unsatisfactory dental restorations, or decubital lesions from poorly fitting removable dentures.

¶The percentages of patients in whom an oral carcinoma developed during follow-up are based on the 74 patients in the total study population who were confirmed to have poor dental hygiene.

‖The percentages of patients in whom an oral carcinoma developed during follow-up are based on the 62 patients in the total study population who had good dental hygiene.

TABLE 2. FOLLOW-UP DATA.

NO. OF BIOPSIES	NO. OF PATIENTS	NO. OF MEN (%)	NO. OF BIOPSY SPECIMENS EVALUATED*	TOTAL NO. OF INITIAL AND FOLLOW-UP VISITS	AVERAGE NO. OF VISITS PER PATIENT	NO. OF PATIENTS WITH CHANGES IN DNA CONTENT DURING FOLLOW-UP
1	54	30 (56)	54	337	6.2	0
2	54	27 (50)	108	414	7.7	1†
3	36	19 (53)	108	379	10.5	1†
4	1	1 (100)	4	6	6.0	0
5	5	4 (80)	25	42	8.4	0
Total	150	81 (54)	299	1178	7.9‡	2

*These specimens were evaluated histologically, and their DNA content was measured.

†The lesions changed from diploid to aneuploid during follow-up.

‡The value is the average number of consultations for the total study population (1178÷150).

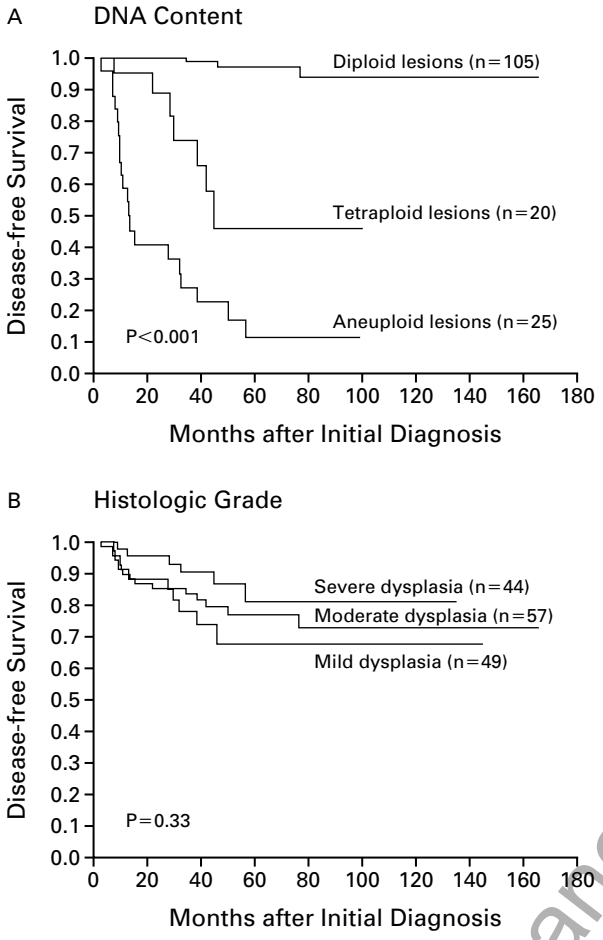

Figure 4. Kaplan–Meier Analysis of the Cumulative Probability of Survival Free of Oral Squamous-Cell Carcinoma, According to the DNA Ploidy (Panel A) and Histologic Grade (Panel B) of the Initial Dysplastic Lesions.

high-risk groups. We did not include patients with previous or concomitant erythroplakia, because erythroplakia carries a high risk of cancer (at least 90 percent).[32] We also excluded patients with previous or concomitant tumors of the upper aerodigestive tract, because multiple lesions may arise as a result of the migration of transformed cells through the aerodigestive tract.[33-35] Thirty-six patients who had already been given a diagnosis of oral carcinoma or carcinoma in situ were also excluded, as such patients are prone to a second carcinoma.[36]

We did include patients in whom the white patches were completely excised at the time of the diagnosis or during follow-up. At least some of these excisions could represent curative measures. Thus, of the 27 patients with aneuploid lesions, the 6 in whom a carcinoma did not develop during follow-up may have been cured by excisional biopsy. If so, the positive predictive value of 84 percent of aneuploidy with respect to the development of a carcinoma and the rate of malignant transformation of 24 percent are underestimates.

The 20 patients with tetraploid lesions represent an intermediate group for which the clinical outcome cannot be reliably predicted by measuring the DNA content. These lesions may be in transition from diploidy to aneuploidy, and aneuploidy might have been observed later on. Additional cytogenetic analysis might improve the predictive value of this group of lesions.[37]

The concept of multiclonal "field cancerization"[38,39] is supported by the fact that patients with oral cancers present with multiple primary tumors or secondary tumors.[36,40] However, multifocal dysplastic lesions could arise from a single site as a result of lateral intraepithelial migration or intraoral dispersion and, with additional genetic changes, acquire a growth advantage.[41,42] The clonal origin of multiple premalignant or malignant lesions in the same patient is supported by recent cytogenetic findings.[43] Either hypothesis — a polyclonal or a monoclonal origin of multiple oral cancers — is consistent with our finding that aneuploidy in only one of several biopsy specimens obtained simultaneously or successively from the same patient can be used to predict the subsequent occurrence of a carcinoma.

In our study, the use of tobacco did not seem to have a confounding effect on the observation that DNA ploidy is a significant prognostic marker in patients with oral leukoplakia. Reliable data on alcohol consumption are difficult to obtain,[44] and such information was not available for almost half the patients in this retrospective study. A confounding effect of alcohol consumption therefore cannot be excluded. Since oral carcinoma develops in relatively few patients with leukoplakia and a history of tobacco use, alcohol use, or both, it is likely that subtle genetic factors also influence the malignant transformation of oral white patches.[45-48] For these reasons, the identification of patients who are in particular need of preventive counseling or active treatment remains a challenge to the clinician.

Whether intensified treatment of aneuploid leukoplakias will reduce the incidence of and rate of death from oral squamous-cell carcinoma is unknown. Current treatments and chemoprevention[49-52] have not significantly improved the poor five-year survival rate of patients with oral squamous-cell carcinoma, perhaps because the intervention comes too late.[4] The increasing incidence of head and neck cancers even among the young[53,54] emphasizes the importance of early identification of the oral white patches that will develop into carcinomas.

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