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ORIGINAL REPORT

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Risk Markers of Oral Cancer in Clinically Normal Mucosa As an Aid in Smoking Cessation Counseling

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A B S T R A C T

Purpose
Quitting smoking may prevent oral cancer. Behavioral intervention to quit smoking may be more efficient if persons are assigned an individual risk of cancer.

Patients and Methods
In this prospective study, we provided counseling and behavioral intervention toward smoking cessation, supplemented by genetic analyses in clinically normal oral mucosa of heavy smokers. Measurement of serum cotinine was used to assess changes in smoking habits.

Results
In cytologic scrapings from 275 heavy smokers with clinically normal mucosa, we found tetraploidy in four and aneuploidy in 19 persons (23 of 275; 8%). Twenty one (91%) of 23 persons with aneuploidy had quit or reduced their smoking habits at the 3-month follow-up, 20 (87%) of 23 persons had done so at 12 months, and 21 (91%) of 23 persons had done so at 24 months. Fifty-one (20%) of the 252 persons without genetic changes in their mucosa had quit or reduced their tobacco habits at the 3-month follow-up, 23 (9%) had done so at 12 months, and 17 (7%) had done so at 24 months ($P < .001$). After 24 months, normalization of DNA content to diploidy was observed in two of four persons with tetraploid (50%), and in 11 of 19 persons (58%) with aneuploid scrapings. One patient developed an oral carcinoma in the floor of the mouth: this patient had an aneuploid scraping obtained 43 months earlier and developed a leukoplakia 28 months before the carcinoma.

Conclusion
Risk markers of oral cancer are present in clinically normal mucosa of heavy smokers, and such findings enhance the adherence to smoking cessation on counseling. Cytogenetic aberrations may normalize after quitting smoking.

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INTRODUCTION

Oral cancer is a lifestyle-related cancer, with tobacco as a primary risk factor.[1-4] Hence, oral cancer is an avoidable disease that may be averted by quitting smoking habits.[5-8] However, tobacco is highly addictive, and quit rates vary to a considerable extent and are predominantly low, with frequent relapses to previous smoking habits.[9,10] Hence, behavioral intervention may become more efficient and its effects of longer duration if counseling aims at conveying information on risk that is more personally relevant to the patient in question.[11-13]

Gross genomic aberrations, such as tetraploidy or aneuploidy in epithelial cells of the oral mucosa, indicates a high risk of oral cancer and cancer-related mortality, at least when it occurs in oral mucosa lesions classified as leukoplakia[14,15] erythroplakia,[16] and even in oral white patches that are classified as being without dysplastic changes on microscopic examination.[17-21] However, oral cancer is the result of a long-standing, multistep process that progresses over several

decades, and the above-mentioned genetic aberrations could conceivably be present also in clinically normal mucosa in persons with risk behavior for oral cancer. If such risk markers could be identified in clinically normal oral mucosa in heavy smokers, early intervention could possibly reverse the cytogenetic changes, with reduced risk of oral cancer. Identification of genetic aberrations in the form of tetraploidy or aneuploidy at such an early stage of disease progression may enhance the effect of smoking cessation as compared with quitting when disease is clinically apparent.[22] Recent studies have shown that cytologic scrapings may be used to detect genetic changes, such as ploidy changes,[23] which are generally accepted as markers of genomic instability, and are most likely a driving force in tumorigenesis.[24]

We undertook this prospective study to determine if genetic markers of cancer risk could be identified in heavy smokers with clinically normal-appearing oral mucosa. We used cytologic scrapings to obtain cells for genetic analysis. We postulated that compliance to the counseling and recommendations given by health care professionals to quit smoking—as assessed through measurement of serum cotinine levels—would increase if we assessed an individually based high risk of acquiring cancer in heavy smokers with no clinically visible lesions in their oral mucosa. Here we present data for the 275 persons included in the study, who all had a minimum of 24 months of follow-up.

PATIENTS AND METHODS

Eligibility

Persons older than 18 years who had smoked 15 or more cigarettes per day for 10 years or more and who had serum cotinine levels equal to or above a value corresponding to heavy smokers (see measurement of serum cotinine levels) at baseline were eligible for the trial. Persons eligible for the study should not have had any previous malignancies or visible lesions, such as oral leukoplakia or erythroplakia, in the oral cavity. All persons included in the study were allowed to attend a smoking cessation program at their own discretion, in addition to receiving counseling at each annual follow-up after the initial 3-month follow-up visit. Persons with known psychiatric disorders were excluded from the study. All participants provided written informed consent.

Study Design

The regional ethics committee of Southern Norway approved the protocol for the study. This prospective, community-based, physician-delivered smoking cessation intervention study was initiated March 1, 2000, and follow-up is still ongoing. Accrual of persons to the trial was stopped in February 28, 2002. Follow-up was done through annual visits to a dentist.

Behavioral Intervention

Behavioral intervention was based on a structured interview with emphasis on history of tobacco and alcohol use. A family history of cancer was also obtained. Study nurses who had been trained specifically gave information on tobacco as a risk factor. The study personnel responsible for counseling on tobacco were not informed about the status of the cytogenetic findings in the scrapings from the oral mucosa. Information on cancer risk from smoking was given at a baseline visit, 3-month follow-up, 12-month follow-up, and thereafter annually. All study participants were informed of the risk for acquiring oral cancer if genetic aberrations in the form of tetraploidy (approximately 25% risk of acquiring oral cancer within 3 years) or aneuploidy (approximately 70% risk of acquiring oral cancer within 3 years) were found in oral leukoplakia. The study participants were explicitly informed that the absence of such genetic aberrations as tetraploidy or aneuploidy was no guarantee that they were not at risk of acquiring cancer in the oral cavity or other parts of the upper aerodigestive tract.

DNA Ploidy Analysis From Cytologic Scrapings

Scrapings were obtained, using wooden spatulas, from the floor of the mouth, and from the ventral and lateral border of the tongue. All investigators received instruction about how to obtain adequate cytologic scrapings. The spatulas were immersed in a preservative solution (Preserv Cyt Solution; CYTYC Corp, Boxborough, MA). Thereafter, cell sampling and processing was done using Thin Prep 2000 (CYTYC Corp). Cytoplasm was removed by enzymatic digestion (type XXIV protease; Sigma Chemical Co, St Louis, MO) for 12 minutes at room temperature. The generation of monolayers and the procedures for DNA ploidy analysis from cytologic scrapings was similar to the procedures on biopsies, and has been published previously.[25-27] Samples that were identified as having aberrant DNA content in cytologic scrapings were reexamined for visible changes in the oral mucosa. If a reexamination revealed visible changes in the oral mucosa, the study participant would be excluded from the study, and a biopsy obtained from the visibly changed oral mucosa.

Measurement of Serum Cotinine Levels

Serum cotinine, which is a biomarker of tobacco smoke exposure, was measured by means of a quantitative competitive enzyme immunoassay that used microtiter plates coated with anticotinine antibodies and detection with a cotinine–horseradish peroxidase conjugate (STC Technologies, Bethlehem, PA). On the basis of previous findings,[28] cutoff levels were used to identify nonsmokers or those passively exposed to tobacco smoke (0 to 24.99 ng/mL), light to moderate smokers (25.00 to 224.99 ng/mL), and heavy smokers ($\geq$ 225.00 ng/mL). Serum cotinine levels were measured at first visit, at the 3- and 12-month follow-up, and thereafter annually.

Negative Controls

As negative controls we selected 25 persons who were younger than 30 years and who had never smoked cigarettes or used chewing tobacco or snuff, and who had only used moderate amounts of alcohol ($<$ 3 units of alcohol per week). Scrapings were obtained from clinically normal mucosa in the same manner as was done in the heavy smokers group and the specimens were analyzed for DNA ploidy status in the same fashion. Negative controls were observed through the same schedule as the study participants.

Statistical Analysis

Frequency and summary data are given whenever appropriate. McNemar's test was used to compare serum cotinine levels before and after behavioral intervention. Serum cotinine levels were chosen as the primary end point because this parameter

represents a robust measure of the recent and long-term tobacco habits of a person. All *P* values are two sided.

RESULTS

Demographic Data

A total of 275 persons were enrolled onto the study. The clinical characteristics of the study participants are shown in Table 1. There were no significant differences between the groups with white lesions compared with the group without visible changes of the oral mucosa. So far, none of the patients have been lost to follow-up. Two of the 275 persons have died of causes other than cancer during the follow-up period. All 275 persons included in the study have been observed for a minimum of 24 months (median, 35 months; range, 24 to 48 months).

Findings in Cytological Scrapings From Clinically Normal Mucosa

None of the 25 control participants were identified with genetic aberrations in the form of tetraploidy or aneuploidy in scrapings from the oral mucosa ($P < .001$). Among the 275 heavy smokers, 19 persons with aneuploid and four persons with tetraploid lesions in clinically normal-appearing oral mucosa were identified. Thus, the incidence of molecularly defined increased risk of oral cancer was 8% in these persons with high-risk behavior for oral cancer, but without any visible changes in their oral mucosa.

Behavioral Modification in Smokers

All 275 persons included in the trial had baseline serum cotinine levels equal to or more than 225 ng/mL. At the 3-month follow-up, 21 of the 23 persons (91%) with findings of tetraploidy (n = 4) or aneuploidy (n = 19) reported that they had quit or reduced their smoking habits (Fig 1A). In 18 persons, the serum cotinine levels were reduced from 225 ng/mL or above, to below 25 ng/mL (Fig 1A, left diagram). Eleven of these (11 of 23; 48%; one in the group with tetraploid scrapings and 10 in the group with aneuploid scrapings) had attended smoking cessation courses in addition to the counseling given by health care professionals at follow-up visits. At the 12-month follow-up, 20 (87%) of the 23 persons with identified genetic aberrations in their scrapings (three with tetraploid scrapings and 17 with aneuploid scrapings) still had quit or reduced smoking (serum cotinine levels < 25.00 ng/mL; $P < .001$; Fig 1B, left diagram). At the 24-month follow-up, 21 persons had quit or reduced smoking (serum cotinine levels < 25.00 ng/mL; Fig 1C, left diagram). In the 252 persons who had no genetic aberrations in their scraping, 201 had not significantly reduced their smoking habits (serum cotinine levels > 225 ng/mL) at the 3-month follow-up (Fig 1A, right diagram). A total of 51 persons (51 of 252; 20%) quit or reduced smoking. Thus, 27 persons (11%) had reduced smoking (serum cotinine levels 25.0 to 224.99 ng/mL, corresponding to light to moderate smokers), and 24 (10%) had quit smoking (serum cotinine levels < 25.00 ng/mL, corresponding to nonsmokers or those passively exposed to tobacco smoke; Fig 1A, right diagram). The corresponding numbers at the 12- and 24-month follow-up were 23 (9%) and 17 (7%), respectively: at the 12-month follow-up, six persons still had reduced their smoking habits, whereas 17 were totally abstinent (Fig 1B, right diagram), and at the 24-month follow-up, five persons still had reduced their smoking habits and 12 were totally abstinent (Fig 1C, right diagram). Among the 252 persons without findings of genetic aberrations in their scrapings, 137 attended a smoking cessation course. All of the 51 persons who have quit or reduced smoking at the 3-month follow-up had attended a smoking cessation course).

Table 1. Baseline Characteristics of Study Population

Characteristic	Heavy Smokers With Clinically Normal Oral Mucosa (n = 275)	
	No. of Participants	%
Sex		
Female	119	43
Male	156	57
Age, years		
30-49	59	21
50-59	118	43
> 60	98	36
Smoking habits, cigarettes/d		
15-24	184	67
> 25	91	33
Cotinine levels, ng/mL		
0-24.99	0	
25-224.99	0	
> 225	275	100

Alterations in Cytogenetic Findings During Follow-Up

Among the 252 persons without genetic aberrations in their scraping at baseline, 15 persons acquired cytogenetic aberrations during the follow-up period: no additional persons after the 3-month follow-up, seven persons (3%) after the 12-month follow-up, and eight additional persons (3%) after the 24-month follow-up (Table 2; Fig 2). All of these persons were among those who continued their tobacco habits. Among the 19 persons with aneuploid lesions at baseline, 11 persons experienced normalization to diploid of the initial cytogenetic aberrations during follow-up: none at the 3-month follow-up, five at the 12-month follow-up, and an additional six at the 24-month follow-up (Table 2; Fig 2). Among the four persons with tetraploid scrapings at baseline, two experienced a reversal of the cytogenetic aberrations, at the 24-month follow-up (Table

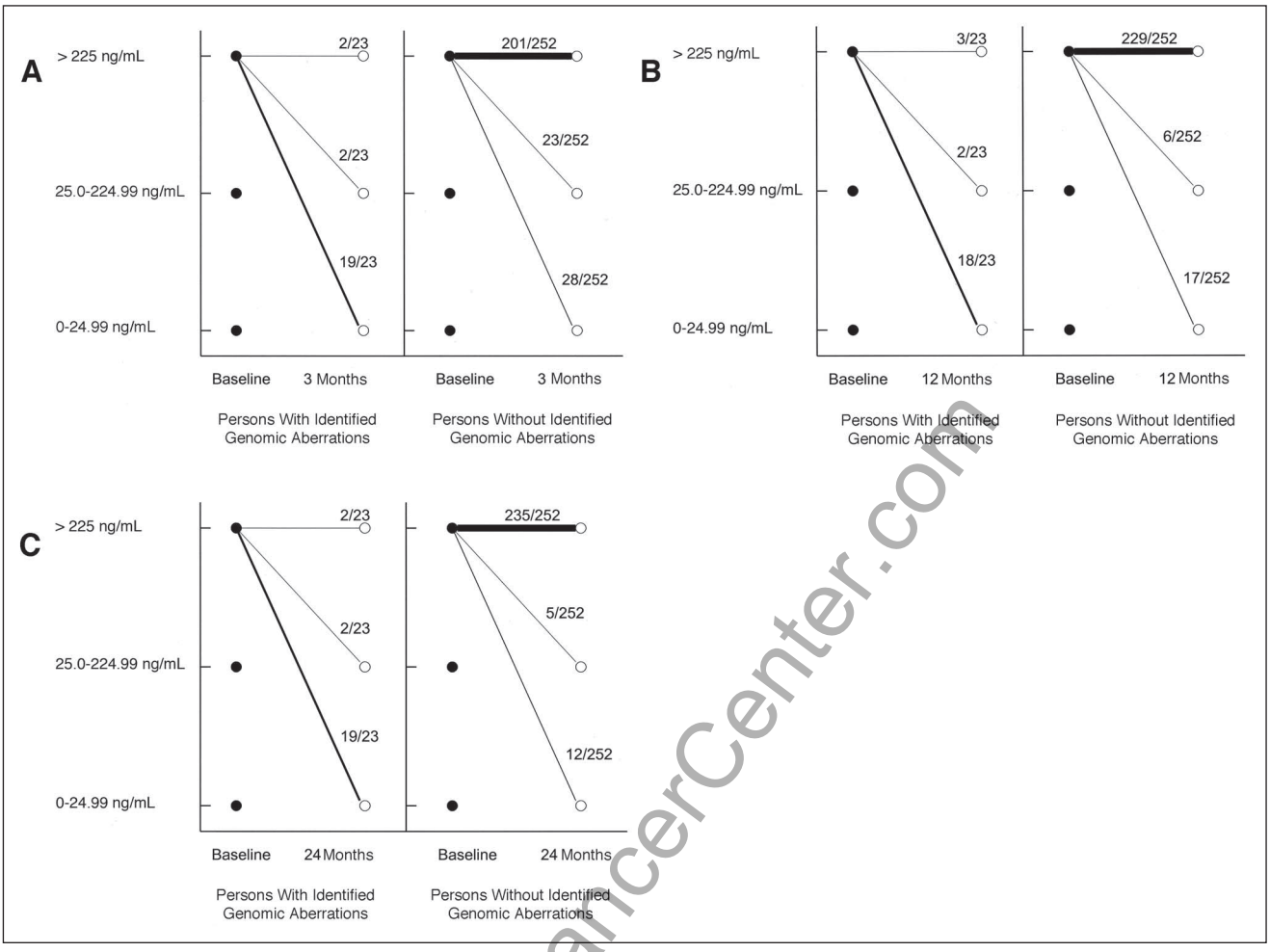

Fig 1. Cotinine levels at baseline, as compared with cotinine levels at the (A) 3-, (B) 12-, and (C) 24-month follow-up.

2; Fig 2). There was no progression to aneuploidy from tetraploidy in the other two persons. Thus, a total of 13 persons (57%) of 23 with initial cytogenetic aberrations at baseline experienced a reversal of the cytogenetic aberrations during follow-up. All of the study participants that experienced a normalization of their initial cytogenetic aberrations had quit smoking and showed serum cotinine levels below 25 ng/mL during the follow-up period ($P < .001$). The smokers who were diagnosed with normal diploid (normal) DNA content in the cells of their scrapings were given the same regimen of counseling as those who were diagnosed with tetraploid or aneuploid scrapings.

Development of Leukoplakia

Eight of the 19 persons (58%) initially diagnosed with aneuploid scrapings and one of the four persons (25%) diagnosed with tetraploid scrapings have developed oral leukoplakia during a median follow-up of 26 months

Table 2. Follow-Up Findings

Ploidy Status	Baseline	3 Months	12 Months	24 Months
Diploid	252	252	245 + 5 normalized aneuploid*	237 + 13 normalized aneuploid or tetraploid*
Tetraploid	4	4	4	2
Aneuploid	19	19	14 + 7 progressed from diploid†	8 + 15 progressed from diploid†
Total	275	275	275	275

*All of the persons with normalized cytogenetic findings in their scrapings had quit smoking.
†All of the persons who acquired cytogenetic aberrations (tetraploidy or aneuploidy) during follow-up continued their smoking habits.

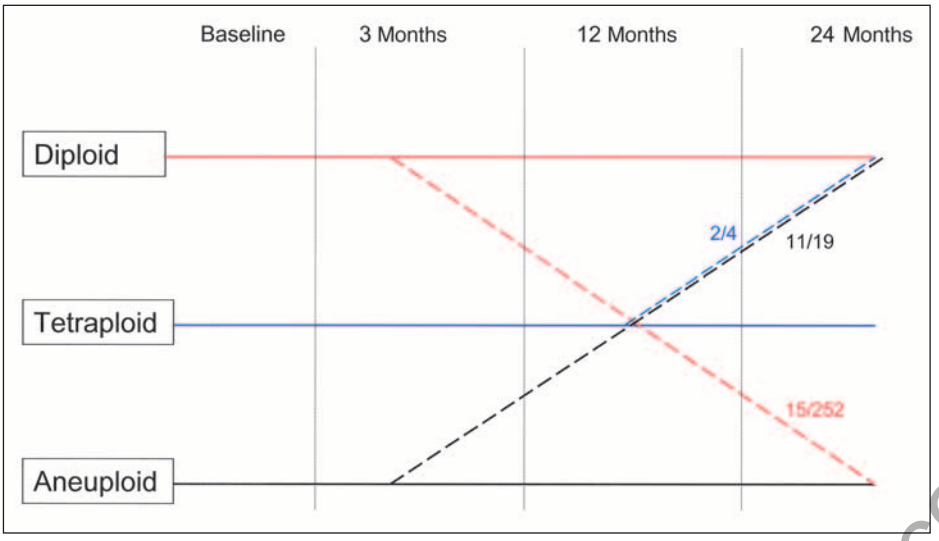

Fig 2. Alterations in cytogenetic findings during follow-up visits. (———) Persons with unaltered findings in cytologic scrapings during follow-up; (– – –) persons with alterations in the cytogenetic findings during follow-up.

(range, 10 to 37 months). None of these leukoplakia occurrences have been diagnosed histologically as carcinoma-in-situ or invasive carcinoma, but dysplasia was found in six of the persons. Ploidy measurements from the biopsies have confirmed the presence of aneuploid or tetraploid cell clones. None of the persons who quit smoking and had a normalization of cytogenetic scrapings, either from tetraploid to diploid, or from aneuploid to diploid, have developed a leukoplakia during follow-up.

Leukoplakia also developed in five (2%) of the 252 persons who initially and later had normal cytogenetic findings in oral scrapings ($P < .001$). However, histologic examination of these lesions has not revealed dysplasia, carcinoma-in-situ, or invasive carcinoma. All lesions were found to be diploid after ploidy measurements on the biopsies obtained after excision.

Malignant Transition of Oral Mucosa

One patient developed an oral carcinoma in the floor of the mouth: this patient had an aneuploid scraping obtained 43 months earlier. The patient developed a leukoplakia 28 months before the carcinoma. Histologic examination of the biopsy obtained after excision of this leukoplakia showed no sign of dysplasia, carcinoma-in-situ, or invasive carcinoma, but ploidy measurement revealed an aneuploid DNA ploidy distribution. This patient had not quit smoking or reduced smoking habits at any time. The baseline cotinine level of this person was 387 ng/mL and was never measured to levels below 300 ng/mL.

DISCUSSION

We demonstrate that markers of genomic instability are present and can be detected by noninvasive methods in heavy smokers with clinically normal-appearing oral mucosa. Because leukoplakia with histologically verified content of dysplasia (which signals an increased risk of oral cancer[29]) subsequently developed in these persons, and because one person with cytogenetic aberrations did develop a carcinoma after developing an aneuploid leukoplakia, it seems that these cytogenetic findings in clinically normal mucosa indicate a high risk of cancer. Informing persons regarding such genetic findings and their significance may enhance compliance to smoking cessation counseling in heavy smokers.[30] The findings from such genetic analyses may guide treatment decisions for these persons, and they support the practice that all persons with high-risk behavior for oral cancer be subjected to analysis of genomic status in their oral mucosa by noninvasive procedures.

The effectiveness of behavioral intervention for smoking cessation is improved when delivered by health care professionals,[31] and high smoking cessation rates can be sustained over a considerable period of time,[32-35] However, counseling persons with high-risk behavior for oral cancer but with no evidence of disease poses a challenge, because counseling must be based on an average risk of contracting cancer, often based on information pertaining to a certain population. Campaigns with provocative and dissonance-arousing appeals seem to improve the effect of smoking cessation campaigns.[36] Nevertheless, behavioral intervention based on epidemiologic findings is far less likely to make an impact on behavior than is an individual risk assessment, even when it comes to counseling regarding a potentially lethal disease such as cancer.[37]

We also present findings indicating that these early genetic changes may be reversed in heavy smokers if they quit their smoking habits. Given the prognostic value of ploidy findings both from this and earlier studies[38-40] it is reasonable to assume that this reflects a reduced risk of oral cancer.

Our study does not rule out the possibility that some aneuploid lesions are overlooked when DNA ploidy status is determined from cytologic scrapings. However, it is difficult to see how other, more invasive means of obtaining tissue specimens could be justified. Therefore, our finding must be considered as an improvement over current approaches, which are clinical inspection and watchful waiting alone—approaches that would not detect such genetic alterations. The approach of watchful waiting may well be accompanied by behavioral intervention, but in persons with no signs of disease, this is likely to give less efficient counseling on smoking cessation.[10,13,35,41] Heavy smokers can now receive definitive counseling based on an individual risk assessment through noninvasive diagnostic procedures, which may be performed at greater liberty than a biopsy.

Given the relative scarcity of oral cancer in the general population, the finding of 23 (8%) of 275 persons with clinically meaningful cytogenetic findings seems high, particularly when one considers that lesions were obtained from clinically normal oral mucosa. Nevertheless, the study participants in our trial were heavy smokers and must be considered to be at high risk of developing oral cancer. The reported frequency of aneuploid epithelial cells in clinically normal-appearing oral mucosa may represent an underestimate, given that scrapings were only obtained from the floor of the mouth and the lateral border of the tongue, and not from other regions of the oral cavity, such as the buccal mucosa. However, this underestimate is not likely to be serious, given that a majority of oral carcinomas arise in the floor of the mouth and the lateral border of the tongue, and therefore most likely are preceded by genetic changes in this region of the oral mucosa. Nevertheless, scrapings may obtain cells mainly from the superficial layers of the epithelium, whereas the cells with genetic aberrations that are detectable through ploidy analysis are predominantly located in the basal layers of the epithelium. Given the robustness of the analysis with respect to interpreting analysis results as diploid, tetraploid, or aneuploid, it is unlikely that our results could represent an overestimate in the frequency of cells with tetraploidy or aneuploidy.

Heavy smokers without ploidy changes could be tempted to continue smoking until ploidy changes are detected in their mucosa. However, all participants were informed that the absence of such genetic aberrations as tetraploidy or aneuploidy was no guarantee that they were not at risk of acquiring cancer, for several reasons. First, ploidy analysis based on cytologic scrapings could potentially have a low sensitivity for detecting such genetic aberrations. Second, there is no reason to believe that oral cancer cannot develop without the presence of ploidy changes. Third, lung cancer is the major tobacco-associated cancer of the upper aerodigestive tract, and there are no data available to indicate that genetic analysis of the oral mucosa may serve as a reliable surrogate marker for the risk of lung cancer, which is substantial in heavy smokers.

The finding that 8% of heavy smokers harbor tetraploid or aneuploid epithelial cells in their mucosa may seem to be a high frequency, and clearly higher frequency than what would be expected given the known prevalence of oral squamous cell carcinoma in the Scandinavian population. Furthermore, the finding that aneuploidy may reverse after smoking cessation is counterintuitive to the extremely high malignant transformation rates observed in aneuploid dysplastic oral leukoplakia.[14,15] Therefore, we speculate that some of the persons with genetic aberrations harbor aneuploidy in epithelial cells other than stem cells. Amplifying cells are regularly shed from the mucosa at intervals ranging from 4 weeks to 12 months,[42,43] and this could reflect some of the reversal of genetic aberrations we observe.

In summary, our findings indicate that genetic risk markers of oral cancer are present in clinically normal oral mucosa of heavy smokers. The identification of such risk markers may be done through noninvasive techniques, and may be used to increase the compliance to counseling regarding smoking cessation. However, these findings pertain to clinically normal oral mucosa, and may not necessarily apply to aneuploid dysplastic oral leukoplakia, which have an extremely high tendency to progress to clinically aggressive cancer within the relatively short time span of 1 to 3 years.[14,15] Therefore, the effect of smoking cessation in heavy smokers with visible oral lesions is unknown.

Authors' Disclosures of Potential Conflicts of Interest

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Consultant/advisory role: Jon Sudbø, Pfizer. Honoraria: Jon Sudbø, Pfizer. For a detailed description of these categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration form and the Disclosures of Potential Conflicts of Interest section of Information for Contributors found in the front of every issue.

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