Advances in Brief

Use of Allelic Loss to Predict Malignant Risk for Low-grade Oral Epithelial Dysplasia[1]

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Abstract

One of the best approaches to identifying genetic changes critical to oral cancer progression is to compare progressing and nonprogressing oral premalignant lesions. However, such samples are rare, and they require long-term follow-up. The current study used the large archive network and clinical database in British Columbia to study loss of heterozygosity (LOH) in cases of early oral premalignancies, comparing those with a history of progression to carcinoma *in situ* or invasive cancer and those without a history of progression (referred to as nonprogressing cases). Each of 116 cases was analyzed for LOH at 19 microsatellite loci on seven chromosome arms (3p, 4q, 8p, 9p, 11q, 13q, and 17p). The progressing and nonprogressing cases showed dramatically different LOH patterns of multiple allelic losses. An essential step for progression seems to involve LOH at 3p and/or 9p because virtually all progressing cases showed such loss. However, LOH at 3p and/or 9p also occurred in nonprogressing cases. Individuals with LOH at 3p and/or 9p but at no other arms exhibit only a slight increase of 3.8-fold in relative risk for developing cancer. In contrast, individuals with additional losses (on 4q, 8p, 11q, or 17p), which appeared uncommon in nonprogressing cases, showed 33-fold increases in relative cancer risk. In conclusion, analysis of LOH at 3p and 9p could serve as an initial screening for cancer risk of early premalignancies. Follow-up investigation for additional losses would be essential for predicting cancer progression.

Introduction

Oral premalignant lesions most often appear clinically as leukoplakia. The criterion for judging the malignant potential of these lesions is based mainly on the presence and degree of dysplasia. Using this criterion, premalignant lesions are classified histologically into stages with increasing risk of developing into invasive SCC,[3] namely: epithelial hyperplasia; mild, moderate, and severe dysplasias; and CIS. High-grade preinvasive lesions (severe dysplasia and CIS) are believed to have a high probability of progression into invasive carcinoma and are therefore treated aggressively (1, 2). However, the majority of the low-grade lesions (mild and moderate dysplasia), as well as hyperplasia without dysplasia, do not progress into cancer (1, 3). Because these early lesions constitute the bulk of oral leukoplakias, and pathohistological stage assignment alone does not predict their malignant potential, a more reliable predictive test needs to be developed.

A central dogma of carcinogenesis is that alteration to critical control genes underlies malignant transformation. Therefore, progressing lesions are likely to be genetically different from their morphologically similar nonprogressing counterparts. The identification of such differences would provide genetic markers useful in predicting the behavior of low-grade lesions. As a result, clinicians would be able to identify which patients with low-grade lesions should be managed more aggressively, either by more frequent screening or by early treatment, using traditional approaches such as surgery or newer techniques such as chemopreventive regimes.

One of the more sensitive techniques available for studying clonal changes in tumors and premalignant lesions is the use of polymerase chain-based microsatellite analysis for allelic loss. The advantage of the procedure is that it requires only small quantities of DNA yet yields valuable data on the loss of chromosomal regions that contain putative suppressor genes. Hence, we can obtain information on critical genetic events even before the identification of the actual suppressor gene. This approach has been used frequently in head and neck cancers (4–10). Studies on premalignant lesions have been limited in number and scope due to the difficulty of obtaining suitable specimens for analysis and due to technical problems associated with working with very small lesions and minute amounts of DNA (11–15). However, frequent occurrence of LOH has been demonstrated in oral premalignant lesions, and several regions of loss common to SCCs have been observed in dysplastic

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[3] The abbreviations used are: SCC, squamous cell carcinoma; LOH, loss of heterozygosity; CIS, carcinoma *in situ*.

lesions and occasionally in hyperplasias (11–16). Califano *et al.* (12) were the first to develop a genetic progression model for head and neck cancer. LOHs at 9p and 3p occur early and are present in hyperplastic or mild dysplasias in addition to higher-grade lesions (11, 12). Other regions of LOH may signal alterations to genes that are more closely related to later events, such as the attainment of immortality (17, 18) or invasion and metastasis, but these associations have only recently begun being explored.

This report describes a genetic study that compared archival premalignant lesions with and without a subsequent history of cancer progression. LOH in hyperplastic and low-grade dysplastic lesions with a known clinical history were examined. The objectives of this study were as follows: (*a*) to characterize the pattern of genetic changes in premalignant lesions by means of LOH analysis using microsatellite markers for the seven chromosomal regions known to be frequently lost in oral tumors (3p, 4q, 8p, 9p, 11q, 13q, and 17p; Refs. 5, 8, 9, 11, and 19); and (*b*) to identify chromosomal differences between premalignancies that would subsequently progress to CIS or SCC and those that would not.

Patients and Methods

Sample Collection. This study used paraffin-embedded archival samples from the provincial Oral Biopsy Service of British Columbia. This centralized Oral Biopsy Service supports dentists and ear, nose and throat surgeons throughout the province, at no cost to the provider or patient. With more than 3500 biopsies of oral lesions collected per year (19 years archived), a large number of patients with early lesions can be followed over time. Cases that progressed into cancer were identified by linking the Oral Biopsy Service database to the British Columbia Cancer Registry, which tracks all histologically confirmed cases of cancer and CIS diagnosed in the province.

Two sample sets were used. The first set consisted of oral lesions from patients with no subsequent history of head and neck cancer. We refer to these cases as nonprogressing cases. The criteria for choosing these samples included a histological diagnosis of hyperplasia or mild or moderate dysplasia, with this diagnosis being confirmed by two pathologists (L. Z. and R. P.) using criteria established by the WHO (2). This set included 54 patients with biopsies of low-grade dysplasia (31 patients with mild dysplasia and 23 patients with moderate dysplasia) and 33 patients with epithelial hyperplasia.

The second sample set, the progressing cases, consisted of 29 patients with hyperplasias or low-grade dysplasias (6 hyperplasias, 9 mild dysplasias, and 14 moderate dysplasias) that later progressed to CIS or SCC. Both the primary hyperplastic or dysplastic lesions and their matching CIS or SCC had to be from the same anatomical site as recorded on pathology reports and patient charts. In addition, the interval between the primary lesions and later CIS or SCC had to be longer than 6 months. The latter criterion was used to exclude cases that might be due to inadequate biopsy.

There was no significant difference between the progressing (*i.e.*, with subsequent clinical history of progression) and the nonprogressing (*i.e.*, without subsequent history of progression) dysplasia sample sets in terms of gender, age distribution, and smoking history (Table 1). However, on average, nonprogressing cases were monitored for over twice the duration (96 *versus* 37 months) to ensure that progression did not occur. Although complete treatment history was not available for all cases, chart review suggested that progressing lesions were treated at least as aggressively as nonprogressing lesions. In British Columbia, low-grade premalignancies are generally excised without a wide margin or followed clinically after an initial incisional diagnostic biopsy. However, persisting or recurring lesions often receive further treatment involving wide excision or chemotherapy. In this study, only 7 of 55 nonprogressing dysplasias were known to be further treated by surgery. In contrast, 13 of 25 progressive dysplasias were known to have had chemotherapy or further excision, of which 6 dysplasias were removed with margin.

Table 1 Characteristics of patients with dysplasia

Features	Nonprogressing cases	Progressing cases	*P*
Age (mean, yrs)	55	58	0.416
Sex (% male)	57	56	1
% with smoking history	85	78	0.170
Follow-up (mean, months)	96	37	0.0001

Tissue Microdissection and DNA Extraction. Areas of hyperplasia, dysplasia, CIS, or tumor were microdissected from sections stained with H&E. The underlying stroma were dissected and used as a source of matched control DNA. The microdissected tissue was digested in 300 μl of 50 mM Tris-HCl (pH 8.0) containing 1% SDS and proteinase K (0.5 mg/ml) at 48°C for 72 h or more. During incubation, samples were spiked with 20 μl of fresh concentrated proteinase K (20 mg/ml) twice daily. The DNA was then extracted as described previously (11). All samples were coded so that LOH analysis was performed without knowledge of diagnosis.

LOH Analysis. The microsatellite markers used for LOH analysis came from Research Genetics (Huntsville, AL) and mapped to the following regions: (*a*) 3p14.2, *D3S1234*, *D3S1228*, and *D3S1300*; (*b*) 4q26, *FABP2*; (*c*) 4q31.1, *D4S243*; (*d*) 8p21.3, *D8S261*; (*e*) 8p23.3, *D8S262*; (*f*) 8p23.3, *D8S264*; (*g*) 9p21, *IFNA*, *D9S171*, *D9S1748*, and *D9S1751*; (*h*) 11q13.3, *INT2*; (*i*) 11q22.3, *D11S1778*; (*j*) 13q12.3–13, *D13S170*; (*k*) 13q14.3, *D13S133*; (*l*) 17p11.2, *CHRNB1*; and (*m*) 17p13.1, *tp53* and *D17S786*. These markers are localized to regions previously shown to be frequently lost in head and neck tumors. The protocol used for LOH analysis has been described previously by Zhang *et al.* (11).

After PCR amplification, PCR products were separated on denaturing polyacrylamide gels and visualized by autoradiography. For informative cases, allelic loss was inferred when the signal intensity of one allele was decreased by at least 50% in the DNA sample from a lesion as compared to the corresponding allele in the matching connective tissue DNA. Samples showing allelic loss were subjected to repeat analysis after a second independent amplification whenever the quantity of DNA was sufficient.

Statistical Analysis. Associations between LOH and progression were examined using Fisher's exact test. Time-to-

Table 2 Allelic loss in progressing and nonprogressing lesions

	Hyperplasia			Low-grade dysplasia		
	Without progression	With progression	*P*	Without progression	With progression	*P*
No. of lesions	33	6		54	23	
No. with LOH[a]	7 (21)	6 (100)	0.001	32 (59)	23 (100)	0.0001
>1 arm lost	0	3 (50)	0.002	17 (31)	21 (91)	<0.0001
>2 arms lost	0	3 (50)	0.002	11 (20)	13 (57)	0.003
LOH on 3p	4/30 (13)[b]	4/6 (67)	0.014	13/53 (25)	14/22 (64)	0.003
LOH on 9p	1/32 (3)	3/6 (50)	0.009	24/52 (46)	19/23 (83)	0.005
LOH on 4q	0/31 (0)	2/6 (33)	0.023	4/48 (8)	6/21 (29)	0.057
LOH on 8p	0/31 (0)	2/6 (33)	0.023	8/51 (15)	11/21 (52)	0.003
LOH on 11q	1/31 (3)	3/6 (33)	0.062	6/52 (12)	9/23 (39)	0.011
LOH on 13q	1/32 (3)	0/4 (0)	1	2/53 (4)	7/21 (33)	0.002
LOH on 17p	0/33 (0)	2/6 (33)	0.020	11/54 (20)	9/22 (41)	0.087
LOH on 3p &/or 9p	5/29 (17)	6/6 (100)	0.004	30/54 (56)	22/23 (96)	0.004
LOH on 3p &/or 9p plus any other arm	0/32 (0)	3/6 (50)	0.002	15/54 (28)	18/23 (78)	0.0001

[a] A total of seven chromosomal arms were tested. Values in parentheses are percentages.
[b] Loss/informative cases (% loss).

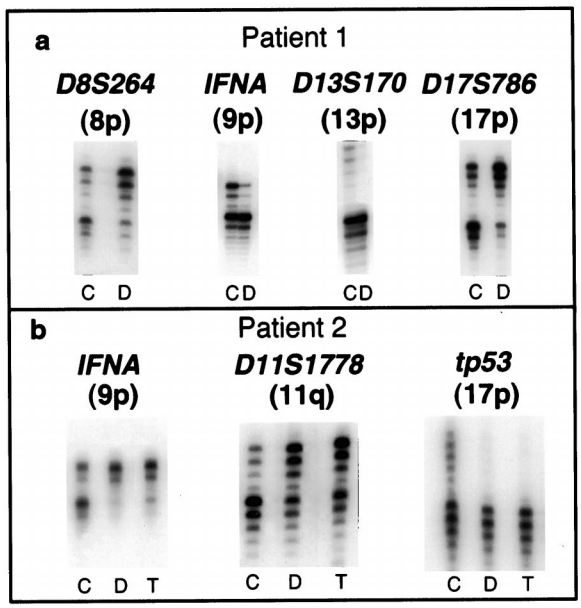

Fig. 1 LOH analysis of two patients (*a* and *b*). DNA was isolated from control stroma (*Lane C*), dysplasia (*Lane D*), or tumor (*Lane T*) microdissected from lesion biopsies. Microsatellite markers, the chromosomal arm being assayed, and patient numbers are indicated *above* each block. *a*, a rare mild dysplasia with multiple allelic loss (loss of the lower allele at *D8S264*, loss of the upper allele at *IFNA*, loss of the upper allele at D*13S170*, and loss of the lower allele at *D17S786*). *b*, patient with a mild dysplasia that later progressed to a SCC. The mild dysplasia shows the same pattern of multiple allelic loss as the tumor (loss of the lower allele at *IFNA* and *D11S1778* and loss of the upper allele at *tp53*).

progression curves were estimated by the Kaplan-Meier method, and comparisons were performed using log-rank test. Clinical differences between progressing and nonprogressing groups were examined using either Fisher's exact test (gender distribution and smoking habit) or an unpaired *t* test (age and follow-up time). $P \leq 0.05$ was considered significant. Relative risks were determined using Cox regression analysis.

Results

Frequency of Allelic Loss. LOH was present in 68 of 116 (59%) premalignant lesions studied, occurring more frequently among dysplastic (55 of 77 lesions, 71%) than hyperplastic (13 of 39 lesions, 33%) lesions ($P < 0.001$). LOH frequencies were dramatically elevated in lesions that later progressed to cancer. All progressing lesions (both hyperplastic and dysplastic) showed LOH at one or more of the 19 microsatellite loci tested. LOH was detected in only 21% of the nonprogressing hyperplasias and 59% of dysplasias.

Multiple chromosomal arm loss was characteristic of progressing lesions (50% of hyperplasia and 91% of dysplasia; see Table 1). It was absent in nonprogressing hyperplasia and occurred in only 31% of the nonprogressing dysplasias.

Pattern of Allelic Loss. The most common losses for both progressing and nonprogressing cases were on 3p and 9p and occurred with a higher frequency in the progressing cases (Table 2). Among nonprogressing cases, 3p and 9p losses were seen in 13% and 3% of hyperplasias and 25% and 46% of dysplasias, respectively. In contrast, 3p and 9p losses were seen in 67% and 50% of the progressing hyperplasias and in 64% and 83% of the progressing dysplasias, respectively.

The frequency of loss at other arms (4q, 8p, 11q, 13q, and 17p) was low for nonprogressing cases. Only 2 of 33 hyperplasias (6%) had loss on any of these arms (one at 11q and one at 13q). Nineteen of 54 (35%) of the nonprogressing dysplasias had loss on these arms, most frequently at 17p (20% of cases) and 8p (15%), followed by 11q (12%), 4q (8%), and 13q (4%).

Further increases in LOH frequencies at 4q, 8p, 11q, 13q, and 17p occurred in lesions that progressed to tumors. For dysplasias, this increase was significant for 8p, 11q, and 13q, and the increase for 4q was of marginal significance ($P = 0.057$). There was also a doubling in the frequency of LOH on 17p (from 20% to 41% of cases), although this increase was not statistically significant ($P = 0.087$). For hyperplasias, increases were significant in comparisons of progressing *versus* nonprogressing lesions for 4q, 8p, and 17p, with 11q being of marginal significance ($P = 0.062$).

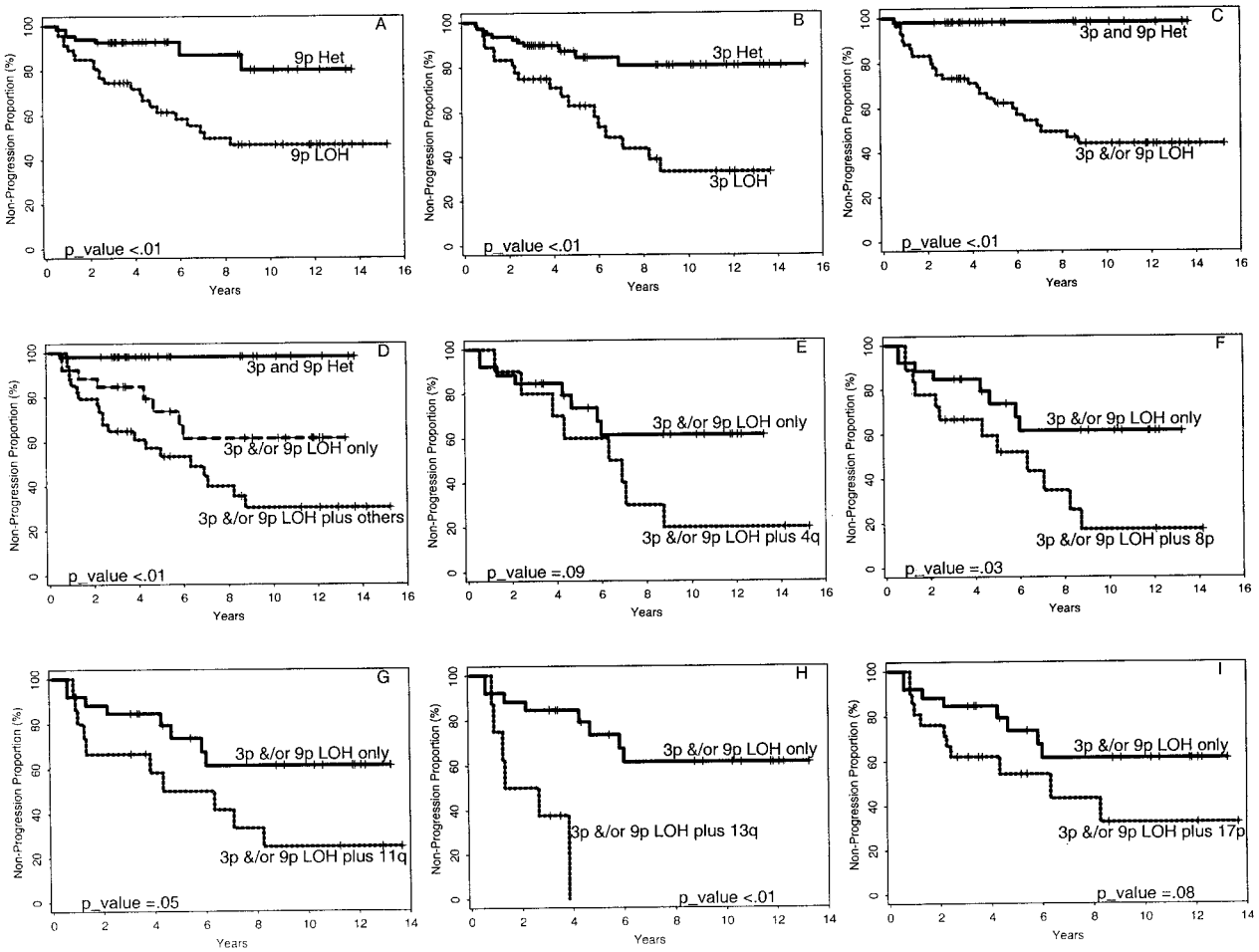

Fig. 2 Probability of having no progression to cancer, according to LOH pattern. *A*, progression as a function of LOH at 9p (no LOH = 69; LOH = 47). *B*, progression as a function of LOH at 3p (no LOH = 80; LOH = 36). *C*, progression as a function of LOH at 3p and/or 9p (no LOH = 56; LOH = 60). *D*, progression as a function of LOH at 3p and/or 9p when this loss occurred in the absence or presence of LOH at any other arm [no additional arms lost = 26; LOH on at least one additional arm (4q, 8p, 11q, 13q or 17p) = 34]. *E–I*, progression as a function of LOH at 3p and/or 9p when this loss occurred with no additional arms lost (n = 26) or with LOH at 4q (n = 10), 8p (n = 18), 11q (n = 15), 13q (n = 8), or 17p (n = 21), respectively.

Comparison of LOH Pattern in Premalignant and Malignant Lesions. Twenty-five of 29 progressing cases had later CIS/SCC biopsies available for LOH analysis. In 17 cases (68%), all allelic losses in the premalignant lesions (the upper *versus* the lower allele) were found in the later lesion (see Fig. 1*b*). In seven of the remaining cases (28%), all but one of the multiple LOHs in the premalignant lesion were present in the tumor. For example, in case 173, the early lesion contained a LOH at 13q that was not found in the later lesion; however, the pair showed loss of the same alleles on 3p, 9p, 8p, and 11q. These data suggest that for most progressing lesions, the later cancer was derived by clonal outgrowth from the earlier lesions.

Progression Risk. Specific LOH patterns in premalignant lesions were examined for association with disease progression by using the Kaplan-Meier method. Because virtually all progressing lesions (28 of 29 lesions, 97%) had LOH on 3p and/or 9p, we tested the value of using these two chromosomes as initial screens for lesions at risk for progression. Time-to-progression curves were plotted as a function of LOH at 9p (Fig. 2*A*), 3p (Fig. 2*B*), or a combination of 3p and/or 9p (Fig. 2*C*). All were significant. An additional comparison was made of cases with loss on these two arms in the presence and absence of LOH at any of the other five chromosomes (4q, 8p, 11q, 13q, and 17p; Fig. 2*D*). A significant difference was again observed. Finally, we separately compared time-to-progression for cases in which 3p and/or 9p LOH was restricted to these two arms alone with cases that had additional losses on each of the chromosome arms (Fig. 2, *E–I*). Significant *P* values were observed for combinations that included 8p, 11q, or 13q.

Additional analyses included an assessment of relative risk of progression for each of the LOH patterns presented in Fig. 2 and a determination of the proportion of cases without progression at 5 years of follow-up. These results are tabulated in Table 3, and an assessment of their significance is given in the "Discussion."

Table 3 Probability of lesions not progressing to cancer after 5 years follow-up

LOH pattern	No. of cases	Proportion (%) of nonprogressing cases (95% CI)[a]	RR (95% CI)
No LOH	48	100	
9p			
9p Het	69	93 (87–99)	1.0
9p LOH	47	61 (48–78)	3.97 (1.68–9.15)
3p			
3p Het	80	85 (76–95)	1.0
3p LOH	36	63 (48–83)	3.74 (1.76–7.93)
3p &/or 9p:			
3p &/or 9p Het[b]	56	98 (95–100)	1.0
All cases with 3p &/or 9p LOH	60	63 (50–77)	24.1 (3.3–176)
3p &/or 9p LOH (but no other arms)	26	74 (57–95)	3.75 (1.32–10.7)
3p &/or 9p LOH (+ LOH at any other arm)	34	53 (38–74)	33.4 (4.48–249)
3p &/or 9p + others			
3p &/or 9p LOH (but no other arms)	26	74 (57–95)	1.0
3p &/or 9p plus 4q LOH	10	60 (36–99)	2.3 (0.86–6.16)
3p &/or 9p plus 8p LOH	18	52 (32–84)	2.59 (1.05–6.37)
3p &/or 9p plus 11q LOH	15	50 (29–86)	2.49 (0.98–6.31)
3p &/or 9p plus 13q LOH	8	0[c]	7.08 (1.93–25.9)
3p &/or 9p plus 17p LOH	21	54 (35–83)	2.2 (0.18–5.5)

[a] CI, confidence interval; RR, relative risk; Het, no LOH or no loss.
[b] Includes eight cases with LOH at other arms.
[c] Calculation does not include two nonprogressing cases that have less than 5 years of follow-up.

Discussion

Linkage of specific patterns of genetic alteration to disease progression is often limited by the difficulty of obtaining clinical specimens from the same lesion over time. In this study, we analyzed microdissected early oral premalignant lesions from 116 patients with or without a history of progression into CIS or invasive SCC for LOH at 19 loci to identify genetic differences between progressing and nonprogressing lesions and to identify genetic profiles that have predictive value for early premalignant lesions.

Can LOH patterns be used as markers to predict risk of progression to cancer? This possibility has been raised by Mao *et al.* (14) on samples collected during a chemoprevention study. Thirty-seven patients with oral leukoplakia were examined for loss at 9p21 and 3p14. LOH at these chromosomal regions was correlated with a greater probability of progression of premalignant lesions into SCC.

The present study showed that progressing and nonprogressing lesions had significantly different LOH profiles, supporting the hypothesis that LOH patterns could be used as cancer risk markers. All progressing lesions showed LOH, characteristically on multiple arms, compared with significantly lower frequencies of loss in nonprogressing cases. Furthermore, all progressing cases (with the exception of one case) in this study showed LOH at 3p and/or 9p. This suggests that loss on these arms is a prerequisite for progression and may be used as an initial screening for assessing cancer risk of oral premalignancies. If LOH at 3p and/or 9p had been used as an initial screening for our study set, without knowledge of LOH at other arms, those cases with 3p and/or 9p LOH would have had a 24-fold increase in the relative risk of cancer progression as compared to those without LOH at either 3p or 9p (Fig. 2*C*; Table 3). However, there was a high frequency of allelic loss on these arms in nonprogressing cases, and because the relative cancer risk for those with LOH limited to 3p and/or 9p was only increased by 3.8-fold, additional markers are essential for better prediction of prognosis.

Our study results suggest that loss at any of the other five chromosomes (4q, 8p, 11q, 13q, and 17p) in addition to LOH at 3p and/or 9p seems to provide a better predictive value. Those cases with such losses had a 33-fold increased risk of progressing to cancer compared to cases that retained both of these arms. Furthermore, time-to-progression curves showed that lesions that had 3p and/or 9p loss with an additional loss on at least one of the indicated arms had a significantly shorter progression time than those with 3p and/or 9p loss only (Fig. 2*D*).

To determine which of the additional losses (on 4q, 8p, 11q, 13q, or 17p) would most significantly increase progression risk, we separately compared those cases with 3p and/or 9p loss alone with those cases with 3p and/or 9p loss plus each of the additional losses (Fig. 2, *E–I*). A significantly shorter time to progression was observed when either 8p, 11q, or 13q LOH was present in addition to 3p and/or 9p LOH. Comparisons with 4q ($P = 0.10$) or 17p ($P = 0.09$) were not statistically significant, although a trend was observed. For each premalignant LOH pattern, the probability of having no subsequent progression is summarized in Table 3. Forty to sixty percent of individuals with additional losses at 4q, 8p, 11q, or 17p developed cancer within 5 years, corresponding to a 2.2–2.6-fold increase in relative risk compared to individuals with only 3p and/or 9p LOH. In contrast, cases with additional 13q loss had a 7-fold increase in risk of progression. Six of the eight cases with loss on this arm had 5 years of follow-up, and all showed progression within this time frame.

In summary, although prospective studies involving large numbers of subjects over time are necessary to fully understand

the relation between chromosomal loss and tumorigenesis, our data suggest that LOH patterns will facilitate the prediction of the malignant potential of low-grade premalignancies. How should this information be used clinically? Patients with LOH at 3p and/or 9p are at risk for progression; their relative risk increases with loss on other arms. Such changes should be a strong signal for active intervention with either traditional or novel forms of therapy such as chemoprevention. On the other hand, patients with 3p and/or 9p loss without changes on the other arms should be at least monitored for further alterations. Because microsatellite analysis can be done on exfoliative cells collected by scraping the surface of these lesions, it should be possible to collect this information noninvasively (4).

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