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3p14 and 9p21 Loss Is a Simple Tool for Predicting Second Oral Malignancy at Previously Treated Oral Cancer Sites[1]

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Abstract

Treatment induces reactive changes that often resemble low-grade dysplasia at former oral cancer sites, complicating histopathological assessment. We tested a set of microsatellite markers shown previously to be predictive of progression for oral premalignant lesions for the ability to predict development of second oral malignancy (SOM). Sixty-eight oral leukoplakia at former cancer sites (with known outcome, 36 progressed to SOM) were evaluated for loss of heterozygosity at 19 loci on seven chromosome arms. 3p and/or 9p loss in these posttreatment leukoplakia was associated with a 26.3-fold increase in risk of developing SOM compared with those that retained both of these arms ($P < 0.001$), with 60% of cases with loss of heterozygosity developing SOM in 2 years. In contrast, histological diagnosis (moderate or severe dysplasia *versus* hyperplasia or mild dysplasia) had only a 1.7-fold increase in risk ($P = 0.11$). The identification of 3p and 9p loss in posttreatment lesions could serve as a simple and direct test for stratifying risk of SOM development.

Introduction

Patients with a history of oral cancer have a high risk of developing a SOM,[4] with up to a third of such patients suffering a recurrence of the tumor or the development of a second primary despite intensive follow-up (1–3). This poor outcome is largely because of a lack of sensitive diagnostic tools for monitoring patients after treatment, with a reliance on clinical and pathological parameters, which are often difficult to assess, for therapeutic decisions. The surgical and radiotherapy treatment that oral cancer patients have received induce reactive white and red lesions at the previous cancer site that are not readily differentiated from (pre)-malignant changes, hence complicating the decision to biopsy. Even when biopsied, treatment-induced reactive changes, which often resemble low-grade dysplasia, may hamper histological assessment of malignant risk. The situation is additionally complicated by the reluctance of clinicians to repeatedly biopsy such fragile sites, because treated tissue also has a reduced capacity to regenerate, hence impeding the timely diagnosis of high-risk changes.

In the search for more reliable prognostic indicators, several investigators have focused on biological molecular markers that might be predictive of SOM development. For example, tumor-specific microsatellite alterations have been detected in the DNA from exfoliated oral cells in saliva of head and neck SCC patients (4) and in scrapes of the oral mucosa of oral cancer patients (5), and gene promoter hypermethylation has been reported in tumors, serum, and saliva of such patients (6, 7). Such studies are still in the early stages with the ability of such indicators to detect lesions at risk for SOM after treatment not yet known. In only a few studies have genetic markers been used on samples collected during the follow-up of patients. Two recent studies used microsatellite analysis to examine oral biopsies from patients after treatment for head and neck SCC (8, 9). The data from both support the potential value of developing this approach to stratify patients at risk of developing SOMs.

Allelic loss has been shown previously to be a strong predictor of progression for oral leukoplakia in patients without a history of oral cancer (10–12) facilitating the classification of morphologically indistinguishable hyperplasia and mild/moderate dysplasias into risk categories (13). In this paper we analyze patterns of allelic loss instrumental in distinguishing risk categories for their ability to predict the development of second oral malignancies from leukoplakia at former cancer sites. We demonstrate the ability of this facile approach to delineate hyperplastic and low-grade lesions with aggressive behavior. Approximately 60% of lesions with such LOH patterns developed second oral malignancies within 2 years.

Materials and Methods

Patients. Criteria for patient inclusion in this study were as follows: (*a*) a histologically confirmed diagnosis of oral SCC or *CIS*; (*b*) treatment with surgery, radiotherapy, or a combination of these therapies, with curative intent; (*c*) development of an OPL within the oral cavity subsequent to treatment at a site that was anatomically contiguous with the primary tumor; (*d*) availability of the OPL biopsy for molecular analysis, with sufficient tissue remaining for molecular analysis; (*e*) availability of pathology reports and patient charts for review; and (*f*) knowledge of outcome. Cases were identified by linking the databases of the BC Cancer Agency and the Vancouver Hospital Health Science Complex (the main treatment centers of oral cancers in the province) to the BC Cancer Registry, which tracks all of the histologically confirmed cases of cancer and *CIS* diagnosed in the province. A total of 68 cases of OPL biopsies at the former cancer site were identified that fulfilled the above criteria. In 36 of these cases (53%), there was development of cancer at the leukoplakia site. These leukoplakia are referred to as those developing "second oral malignancy" and are called "SOM" cases. In the remaining 32 cases (47%), an oral leukoplakia developed at a former cancer site but did not progress into cancer (termed "non-SOM"). It should be noted that this SOM rate does not reflect the frequency in the general population of BC, because it is specifically restricted to those cases that develop a leukoplakia at a former cancer site that is subsequently biopsied. The current rate for SOM in an ongoing longitudinal trial at the Vancouver Clinic is ~30%,[5] which is consistent with reports of other populations (1–3).

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[4] The abbreviations used are: SOM, second oral malignancy; *CIS*, carcinoma *in situ*; LOH, loss of heterozygosity; SCC, squamous cell carcinoma; OPL, oral premalignant lesion; BC, British Columbia; SPT, second primary tumor; CI, confidence interval; RR, relative risk.

[5] Unpublished observations.

This study did not determine whether or not SOMs were a tumor recurrence or SPT. Because all of our tumors occurred at the same site as the primary SCC, the classification of SPT would be based on the occurrence of the SOM at >3 years from the primary SCC, using the classification criteria of Warren and Gates (14). Because there is no difference in the proportion of cases with LOH at 3p and/or 9p that developed into SOMs before and after 3 years, we concluded that this genetic pattern applies to both SPTs and recurrent tumors.

Tissue Microdissection and DNA Extraction. Diagnoses were confirmed by two pathologists using criteria established by the WHO (15). Areas of hyperplasia, dysplasia, *CIS*, or tumor were isolated by microdissection along with the underlying stroma for each case as a source of matched control DNA. Protocols for digestion and extraction of samples are as described previously (16). All of the samples were coded so that LOH analysis was performed without knowledge of diagnosis.

LOH Analysis. The microsatellite markers used for LOH analysis came from Invitrogen (Burlington, Ontario, Canada) and mapped to the following regions: 3p14.2 (*D3S1234*, *D3S1228*, and *D3S1300*), 4q26 (*FABP2*), 4q31.1 (*D4S243*), 8p21.3 (*D8S261*), 8p23.3 (*D8S262*), 8p23.3 (*D8S264*), 9p21 (*IFNA*, *D9S171*, *D9S1748*, and *D9S1751*), 11q13.3 (*INT2*), 11q22.3 (*D11S1778*), 13q12.3–13 (*D13S170*), 13q14.3 (*D13S133*), 17p11.2 (*CHRNB1*), and 17p13.1 (*tp53* and *D17S786*). These markers are localized to regions shown previously to be frequently lost in head and neck tumors, and are the same as those used in an earlier study of cancer progression of primary oral dysplasia by this laboratory (13). The protocol used for LOH analysis is described in Zhang *et al.* (16).

After 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 with 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. Differences and associations between SOM and non-SOM groups were examined using either Fisher's exact test for categorical variables (gender, smoking habit, stage, grade, and LOH) or *t* test for continuous variables (age and follow-up time). Time-to-SOM curves were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. RRs and the corresponding 95% CIs were determined using Cox regression analysis. All of the tests were two-sided. $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Clinicopathological Characteristics and SOM. Frequency statistics for clinicopathological characteristics of the 68 patients in this study are shown in Table 1. The patients included a near-equal number of males and females (51% male), with the majority (91%) being smokers (43 of the 62 cases had this information available). The average age was 58 (18–90 years). The majority of the tumors (56 of 68, 82%) were located at high-risk cancer sites (the floor of the mouth, the ventrolateral tongue, and the soft palate complex). Twenty-two percent of tumors were *CIS*, 49% were stage I or II, and 29% were stage III or IV. Ninety-two percent of the invasive tumors were well or moderately well differentiated, with 8% poorly differentiated. Treatment was by surgery for 38% of cases, with radiotherapy for 40% of cases, and with a combination of these therapies for 22% of cases.

Table 1 *Clinicopathological features of patients*

	All cases (%)	Cases with SOM (%)	Non-SOM (%)	P^a
Total	68 (100)	36 (100)	32 (100)	
Mean age (yr) ± SD	58.3 ± 14.7	58.4 ± 15.9	58.1 ± 13.6	0.93
Male sex—no. (%)	35 (51)	17 (47)	18 (56)	0.48
Tobacco use ever—no. (%)	43 (69)[b]	22 (63)	21 (78)	0.27
Stage				
CIS (stage 0)	15 (22)	7 (19)	8 (25)	0.77
I and II	33 (49)	19 (53)	14 (44)	
III and IV	20 (29)	10 (28)	10 (31)	
Histological grade (for 53 invasive SCC)				
Well or moderately well differentiated	49 (92)	27 (96)	22 (88)	0.33
Poorly differentiated	4 (8)	1 (4)	3 (12)	
Mean follow-up time (months) ± SD	83 ± 80	64 ± 62	104 ± 93	0.04

[a] *P* was calculated comparing SOM *versus* non-SOM patients.
[b] Smoking information available for 35 SOM and 27 non-SOM cases.

These clinicopathological features were compared in cases in which the leukoplakia at the former cancer site progressed into an SOM (SOM cases) and those without progression (non-SOM cases; Table 1). No significant association was noted between development of SOM and gender, age distribution, and smoking history (Table 1; all have $P > 0.05$). Nor were the stage, grade, location of lesion, or type of treatment of the primary cancer significantly different in SOM and non-SOM cases. It is important to note that on average, cases that did not develop SOM were monitored for more than twice as long as those that did (104 *versus* 64 months on average; $P = 0.04$). Also, chart examination suggests that the development of SOM in the SOM cases was not because of a preferential treatment of the leukoplakia in non-SOM cases. In fact, 44% of leukoplakia that subsequently developed SOM had a wide excision or chemotherapy compared with 13% of leukoplakia in the non-SOM group, suggesting that SOM leukoplakia were actually more aggressively treated. On the basis of chart review there was no apparent reason for the more aggressive treatment of the SOM group. There is no significant difference in the site of lesions for the two groups. Because this is a retrospective study, the recording of other parameters such as size is incomplete, although available information shows no difference for the two groups. These parameters will have to be assessed more completely in perspective studies.

Histology of Leukoplakia and SOM. The majority of the 68 post-SCC treatment leukoplakias in this study were either hyperplasia (27 cases, 40%) or mild dysplasia (20 cases, 29%), with 14 cases of moderate dysplasia (21%) and 7 cases of severe dysplasia (10%). OPLs with moderate and severe dysplasia at previous oral SCC sites are believed to have a high cancer risk and are generally removed. Our study results support an aggressive treatment of these lesions as the majority of leukoplakia with moderate and severe dysplasia (14 of 21 cases, 66%) progressed into cancer.

On the other hand, there is no clear agreement on the management of hyperplasia and mild dysplasia, which represent the most difficult category of OPLs for risk assessment. Hyperplasia and mild dysplasia are considered, by many, to be low risk, and they usually do not invoke a decision to treat. However, our data showed that close to half of the OPLs (22 of 47 cases, 47%) without dysplasia or with minimal dysplasia progressed into SOM. Two inferences could be drawn from such results. First, the data suggest that clinical appearance of OPLs at the previous oral SCC site signals a significant cancer risk even in the absence of dysplasia or with minimal dysplasia. Second, better risk prediction markers are needed to better stratify the half of the low-grade high-risk lesions from the other half of morphologically similar low-risk lesions.

LOH Frequencies and SOM. Although LOH was a frequent event in all of the oral leukoplakia that developed at former cancer sites (present in 53 of 68 cases, 78%), such frequencies were elevated significantly in lesions that later developed into cancer. Thirty-five of 36 of these lesions (97%) showed LOH at 1 or more of the 19 microsatellite loci tested, compared with 15 of 32 (47%) lesions that did not progress to cancer ($P = 0.0001$; Table 2). In 26 of the 36 SOM cases (72%), LOH was observed at multiple chromosomal arms, compared with 9 of 32 (28%) non-SOM cases ($P = 0.0006$; Table 2). These data suggest that high LOH frequencies in posttreatment leukoplakia are associated with SOM.

The most common losses for both SOM and non-SOM cases were on 3p and 9p with a higher frequency in the SOM cases (Table 2).

Table 2 *Allelic loss in leukoplakia that developed into SOM compared to that which did not*

	SOM (%) (n = 36)	Non-SOM (%) (n = 32)	P
No. with LOH[a]	35 (97)	15 (47)	0.0001
>1 arm lost	26 (72)	9 (28)	0.0006
LOH on 3p	22/35 (63)[b]	3/32 (9)[b]	0.0001
LOH on 9p	28/36 (78)	9/32 (28)	0.0001
LOH on 4q	12/30 (39)	1/26 (4)	0.0014
LOH on 8p	13/36 (36)	6/32 (19)	0.18
LOH on 11q	6/32 (19)	1/31 (3)	0.10
LOH on 13q	4/32 (13)	2/30 (7)	0.67
LOH on 17p	13/36 (36)	9/32 (28)	0.61
LOH on 3p and/or 9p (but no other arms)	11/36 (31)	3/32 (9)	0.039
LOH on 3p and/or 9p (plus LOH at any other arm)	24/36 (67)	7/32 (22)	0.0003
All cases with LOH on 3p and/or 9p	35/36 (97)	10/32 (31)	0.0001

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

Sixty-three percent of these cases had LOH on 3p and 78% on 9p, compared with 9% and 28%, respectively, of non-SOM cases (P = 0.0001). The frequency of loss at other arms (4q, 8p, 11q, 13q, and 17p) was low for non-SOM cases. Eleven of 32 (34%) non-SOM leukoplakia had loss on these arms, most frequently at 17p (28% of cases) and 8p (19%), followed by 13q (7%), 4q (4%), and 11q (3%). Increases in LOH frequencies at 4q, 8p, 11q, 13q, and 17p occurred in leukoplakia of SOM cases. However, this increase was significant only for 4q (P = 0.001), with the increase for 11q of marginal significance (P = 0.10). Strikingly, when 3p and 9p loss were considered together, virtually all of the SOM cases (97%) showed a LOH on 3p and/or 9p.

Time to Outcome for Different LOH Patterns. Specific LOH patterns in the posttreatment leukoplakia were examined for association with SOM development by using the Cox proportional hazard regression method, and the "time-to-SOM" curves were estimated by the Kaplan-Meier method. First, each arm was evaluated separately. Time-to-development of SOM was significantly decreased for cases with LOH at 3p, 9p, 4q, 8p (Fig. 1, *A–D*; P < 0.001), and 11q (P = 0.0016; data not shown), but not significant for 13q or 17p (Fig. 1, *E* and *F*). We also tested the following LOH combinations: (*a*) 3p and/or 9p, because 35 of 36 SOM cases showed this pattern (Fig. 1*G*); and (*b*) cases with loss on these two arms in the presence of and (*c*) in the absence of LOH at any of the other 5 chromosomes (4q, 8p, 11q, 13q, or 17p; Fig. 1*H*). All three of these patterns were associated with a decrease in time-to-SOM. Finally, as a comparison with the LOH data (Fig. 1, *A–F*), we determined whether histological diagnosis of the leukoplakia affected time to outcome. As shown in Fig. 1*I*, there was an increase in time-to-SOM in moderate and severe dysplasia when compared with hyperplasias and mild dysplasias, but this increase was not significant (RR = 1.7; P = 0.11).

An assessment of RR of SOM development was made for each of the above LOH patterns (Table 3). Risk was 4.2-fold higher for cases with 3p LOH compared with 3p retention (95% CI, 2.1–8.6) and 3.8-fold higher for 9p LOH compared with 9p retention (95% CI, 1.7–8.5). RR for 4q, 8p, and 11q was <3 with RR for 13q and 17p at <1.2. In contrast to these results with molecular markers, histological diagnosis for the leukoplakia gave only a 1.7-fold increase in risk when high-grade lesions (moderate/severe dysplasias) and lesions with little or no dysplasia (hyperplasia/mild dysplasia) were compared.

A striking increase in RR was observed when loss at 3p and 9p

Table 3 *RRs (95% CI) of leukoplakia developing into a SOM*

	RR (95% CI)	P
LOH pattern		
3p Het *vs.* LOH	4.23 (2.1–8.5)	<0.001
9p Het *vs.* LOH	3.77 (1.7–8.4)	<0.001
4q Het *vs.* LOH	2.61 (1.2–5.5)	0.008
8p Het *vs.* LOH	2.49 (1.2–5.1)	0.01
11q Het *vs.* LOH	2.85 (1.2–7.0)	0.016
13q Het *vs.* LOH	1.06 (0.37–3.0)	0.92
17p Het *vs.* LOH	1.18 (0.60–2.3)	0.62
3p and/or 9p Het *vs.* LOH		
3p and/or 9p LOH (no other arms)	23.5 (3.0–183)	<0.001
3p and/or 9p LOH (plus LOH at any other arm)	25.4 (3.4–188)	<0.001
All cases with 3p and/or 9p LOH	26.3 (3.2–193)	<0.001
Histological diagnosis		
Hyperplasia or mild dysplasia *vs.* moderate or severe dysplasia	1.7 (0.87–3.4)	0.11

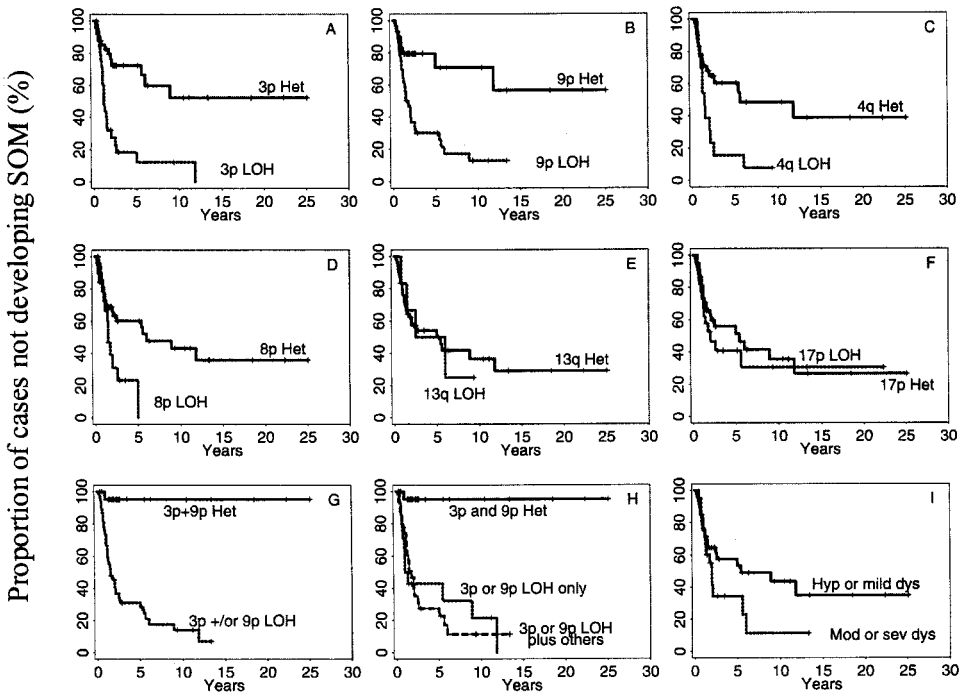

Fig. 1. Probability of development of a SOM from leukoplakia at former cancer site, according to LOH pattern and histology. *A*, progression as a function of LOH at 3p (no LOH = 42; LOH = 25). *B*, progression as a function of LOH at 9p (no LOH = 31; LOH = 37). *C*, progression as a function of LOH at 4q (no LOH = 42; LOH = 13). *D*, progression as a function of LOH at 8p (no LOH = 49; LOH = 19). *E*, progression as a function of LOH at 13q (no LOH = 56; LOH = 6). *F*, progression as a function of LOH at 17p (no LOH = 46; LOH = 22). *G*, progression as a function of LOH at 3p and/or 9p (retention of both arms = 23; LOH of either arm = 45). *H*, 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 (retention of 3p and 9p = 23; no additional arms lost = 14; LOH on at least 1 of the following arms: 4q, 8p, 11q, 13q, or 17p = 31). *I*, progression as function of LOH for leukoplakia with histological diagnoses of hyperplasia or mild dysplasia compared with moderate or severe dysplasia (hyperplasia/mild dysplasia = 47; moderate or severe dysplasia = 21).

were combined. Cases with LOH at 3p and/or 9p had a 26.3-fold increase in risk ($P < 0.001$) compared with those that retained both arms. This suggests that allelic loss at 3p14 and/or 9p21 may be an extremely effective predictor of cancer development at previously treated oral cancer sites. It is of interest that knowledge of loss at other arms does not improve predictive value for this comparison. When cases with LOH at 3p and/or 9p only were compared with those with 3p and/or 9p combined with loss at any other arm (4q, 8p, 11q, 13q, or 17p) the increase was not significant (RR = 1.02; 95% CI, 0.49–2.11).

In summary, this work describes for the first time the use of microsatellite analysis to predict outcome for leukoplakia developing at former cancer sites. The data suggest that 3p and 9p loss in these OPLs is a strong risk indicator of SOM development, regardless of the histological diagnosis. Twenty-two (61%) of the lesions that developed into SOM were histologically benign (hyperplasia or mild dysplasia) and, as such, would probably have been left untreated by most clinicians. However, 21 of these lesions (95%) had this high-risk molecular profile. Also of note is the speed at which such leukoplakia progress. Of 45 patients with LOH at 3p and/9p, it was estimated that 60% (95% CI, 42–73) developed into SOM within 2 years and 69% (95% CI, 50–80) at 3 years. At 5 years, the proportion increased to 72% (95% CI, 53–83). These data strongly suggest that the identification of such alterations at a former cancer site should alert the clinician to the presence of a potentially aggressive lesion, even if the histological diagnosis is hyperplasia or mild dysplasia, and even if distinction between SPT and recurrence could not be determined.

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