

Allelic Losses in OraTest-directed Biopsies of Patients with Prior Upper Aerodigestive Tract Malignancy¹

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ABSTRACT

Genetic alterations at critical chromosome loci have been shown to be predictors of the progression of oral premalignancy-to-invasive cancer. We obtained a unique group of oral biopsies, initially collected during a prospective study designed to test the ability of OraTest (toluidine blue), to identify recurrent oral neoplastic lesions in patients with definite therapy for head and neck or upper aerodigestive tract (UADT) cancer. A total of 46 cases, including 13 squamous cell carcinoma (SCC), 11 carcinoma-*in situ* or dysplasia, and 22 morphologically normal oral biopsies, were analyzed for loss of heterozygosity (LOH) at 9p21, 3p21, and 17p13(TP53) by microsatellite analysis. LOH at one or more tested markers in at least one biopsy was detected in 76% (35 of 46) cases. All of the SCC and carcinoma-*in situ* cases showed LOH, and, strikingly, more than one-half (69%, 13 of 22) of morphologically normal epithelia also harbored LOH in at least one tested marker. The most frequent LOH was found on chromosome 9p21 (69%, 31 of 45). LOH was observed at 3p21, 17p13(TP53), or in multiple chromosomal arms significantly more often in SCC than in normal epithelia. In the majority of cases, two oral biopsies, one from an OraTest-staining positive area and another from a negative area adjacent to the stain, were collected. Among 25 LOH positive cases with two biopsies, identical allelic losses were confirmed between stained and non-stained biopsies in 16 cases. In the remaining nine cases with discordant LOH patterns between two biopsies, eight cases showed LOH at more genetic loci in OraTest-stained areas. Our data confirm that clonal genetic alterations, especially 9p21 deletion, are often present in the oral epithelia of

patients with previous UADT malignancy and, combined with previous studies, suggest that genetic analysis will help stratify patients at risk of developing a secondary oral cancer. In addition to detecting cancer, our study suggests that OraTest can detect clinically occult lesions in the progression pathway to oral cancer.

INTRODUCTION

The risk of developing a second oral malignancy is high in patients with a previous history of oral or UADT³ cancer. Several studies have suggested that this risk significantly increases over time and may approach a 2.2- to 19.0-fold risk over that of the general population (1–3). Other studies have independently reported that almost one-third of patients who suffer from a previous oral or UADT malignancy will eventually develop a secondary cancer in the oral cavity (4, 5). The secondary cancers in these patients may represent a recurrence or a second primary malignancy in the oral cavity, lung, and other sites (5). The majority of patients develop recurrence within 1–2 years after treatment for the primary cancer.

The reason for the risk has been attributed to a concept termed “field cancerization,” presumably caused by the consumption of tobacco and alcohol in these patients (6). Patients with field cancerization may harbor patches of dysplastic or premalignant changes throughout the aerodigestive tract. It is thought that these patches represent nascent cancers in the early stages of clinical presentation. Recent molecular studies have shown that tumors occurring at the original site or even at a substantial distance away from the primary cancer are often clonally related (7–9). Because only a portion of these patients will develop a second oral malignancy, it is important to identify and characterize the genetic alterations that lead to cancer formation in these lesions. These genetic alterations may represent important markers that enable us to identify patients at risk for the development of second cancers and/or predict a specific response to various available therapies.

Recent allelotype studies have revealed frequent losses or imbalances at several chromosomal arms in head and neck cancer (10, 11). Many studies have confirmed that a specific region at 9p21 is the most frequent target of LOH in head and neck cancer (>70% of informative tumors) targeting inactivation of the tumor suppressor gene, *p16INK4A/CDKN2A* (12–14). Chromosomal arms 3p, 11q, and 17p harbor other loci frequently lost in head and neck cancer, showing LOH in more than 50% of informative tumors (10, 15). Moreover, all of the aforementioned, chromosomal alterations have been demonstrated to be frequent and relatively early events in the progres-

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³ The abbreviations used are: UADT, upper aerodigestive tract; CIS, carcinoma-*in situ*; LOH, loss of heterozygosity; SCC, squamous cell carcinoma.

Table 1 Frequency of LOH in oral biopsy from the patients with a previous oral or UADT malignancy^a

	3p21	9p21	TP53	Multiple loss	Total loss
SCC	60 (6/10) ^b	91 (11/12)	55 (6/11)	69 (9/13)	100 (13/13)
CIS/Dysplasia	13 (1/8)	82 (9/11)	40 (2/5)	18 (2/11)	82 (9/11)
Nonneoplastic	14 (2/14)	50 (11/22)	8 (1/12)	5 (1/22)	59 (13/22)
Total	28 (9/32)	69 (31/45)	32 (9/28)	26 (12/46)	76 (35/46)

^a Difference of LOH frequency at different loci among three groups was statistically tested by Fisher's exact test (two-sided). Significance of *P* is further described in "Results."

^b Percentage of LOH (number of cases with LOH/number of informative cases).

sion of head and neck cancer (12, 16). More recently, retrospective studies have shown that LOH at 9p21 and 3p is closely associated with the phenotype and clinical behavior of oral premalignant lesions (17, 18). Moreover, LOH at 17p or *p53* mutations appeared to convey a markedly increased risk of cancer progression in these lesions. Further characterization of these early losses may identify specific markers able to predict the risk of progression in oral premalignancy (19, 20).

In this study, we analyzed microsatellite markers for LOH at 9p21, 3p21, and 17p (TP53) in oral biopsies from patients with a previous head and neck or UADT cancer. These biopsies are unique in that they were obtained during a prospective, double-blind IND clinical study designed to test the ability of OraTest to identify premalignant and cancerous lesions in patients with definite therapy for a previous UADT cancer. We found clonal genetic changes in all of the CIS lesions and invasive cancers. Moreover, we found LOH in at least one marker in two-thirds of the dysplastic or morphologically-normal-appearing biopsies. Our observations confirm previous studies and help establish these microsatellite markers as supplements to histological and clinical assessment. These results also suggest that OraTest can detect clinically occult lesions in the progression pathway to oral cancer. Prospective longitudinal studies, currently underway, are needed to establish the relative risk of secondary oral cancer in patients with previous head and neck cancer based on OraTest directed biopsies and molecular analysis.

MATERIALS AND METHODS

Patients. Biopsies from 81 patients previously diagnosed with oral or UADT malignancy were taken during the original study, and 46 cases were available for molecular analysis. Of the 35 cases not available, tissue blocks from 16 patients were not forwarded to us by the participating centers, and tissue from the other 19 cases had insufficient material for further analysis. All of the patients were over 18 years old. The last surgical, radiological, or chemotherapy treatment was performed at least 3 months, but not more than 2 years, before entry of the patient in the study and provided informed consent. Oral mucosa biopsies were performed in patients with the aid of OraTest (toluidine blue), which stains malignant cells and normal epithelia differentially and helps to visualize neoplastic lesions in the mucosa. Biopsies were preferentially taken from an OraTest-positive area if only one sample was collected, but in the majority of patients, two punch biopsies, one from an OraTest-positive area and another from an adjacent negative staining area (not more than 5 mm away from the margin of stain), were taken. The size

of each biopsy was 3–4 mm. In all, 80 biopsies from 46 patients were available after being immediately fixed in 10% formalin and processed routinely. Tissues were stored as paraffin-embedded materials. Histopathological morphology of the biopsies was initially evaluated by several pathologists from the participating hospitals and confirmed by W. H. W. at Johns Hopkins Hospital.

Microdissection and DNA Preparation. Fifteen to 35 sections (10- μ m) were cut from each tissue block. One section was stained with H&E and served as a reference for microdissection. Microdissection was performed on the additional unstained sections. Mucosal epithelia were meticulously dissected from the sections and collected. Subsequently, microdissected samples were placed in xylene overnight to remove the paraffin, pelleted in 70% ethanol, dried, and incubated in 1% SDS/proteinase K (1 mg/ml) at 58°C for 48 h. Digested tissues were then subjected to phenol-chloroform extraction and ethanol precipitation as described previously (21). Nonneoplastic stroma of the biopsy from each case were processed simultaneously in the same way as epithelia and served as a normal control for allelotyping.

Analysis of LOH. Four microsatellite markers were selected for analysis. The selected markers are located at chromosome loci 3p21 (*D3S1067*), 9p21 (*D9S171* and *D9S736*), and 17p13 (*TP53*). These regions have been shown to be the most frequently deleted in head and neck cancer. Primers for microsatellite amplification were obtained from Research Genetics (Huntsville, AL). A forward primer from each pair was end-labeled with [γ -³²P]ATP (20 mCi/ml; Amersham) and T4 kinase (New England Biolabs) in a total volume of 50 μ l. A 2- μ l sample of DNA (~20ng) was amplified in a total volume of 10 μ l containing 1 \times PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris, 6.7 mM magnesium chloride, 10 mM B-mercaptoethanol, 1% DMSO, and 1.5 mM deoxynucleotide triphosphates), 20 ng of each unlabeled primer, 0.2 ng of labeled forward primer, and 1 unit of Taq polymerase. Amplification was carried out for 35 cycles consisting of 95°C 30 s for denaturation, 55–60°C 45 s for annealing, and 72°C 1 min for extension. Three μ l of PCR product were separated on an 8% denaturing polyacrylamide gel and exposed to film for 12–48 h. In informative cases, allelic losses were recorded when the signal intensity from one allele was decreased by more than 50% in the epithelial sample as compared with the signal from the same allele in the normal control DNA (12). All of the losses were recorded by two independent observers (Z. G. and D. S.), and no discrepancies were observed.

Case No.	Sample and Histology		9p21	3p21	17p13
Cases with SCC					
1	B N	SCC SCC	■	■	■
2	B N	SCC SCC	■	■	■
3	B	SCC	■	■	■
4	B N	SCC LDPL	■	■	■
5	B N	SCC SCC	■	■	■
6	B N	SCC SCC	■	■	■
7	B N	SCC No atypia	■	■	■
8	B N	SCC HDPL	■	■	■
9	B N	SCC SCC	■	■	■
10	B N	SCC HDPL	■	■	■
11	B N	SCC LDPL	■	■	■
12	B	SCC	MI	■	■
13	B N	SCC No atypia	■	■	■
Cases with CIS/ dysplasia					
14	B N	LDPL No atypia	■	■	■
15	B	LDPL	MI*	MI	■
16	B	CIS	■	■	■
17	B	CIS	■	■	■
18	B N	LDPL LDPL	■	■	■
19	B	LDPL	■	■	■
20	B	LDPL	■	■	■
21	B	LDPL	MI*	■	■
22	B N	LDPL LDPL	■	■	■
23	B N	LDPL LDPL	■	■	■
24	B N	LDPL No atypia	■	■	■
Cases without neoplastic morphology					
25	B N	No atypia No atypia	■	■	■
26	B N	No atypia No atypia	■	■	■
27	B N	No atypia No atypia	■	■	■
28	B	No atypia	■	■	■
29	B N	No atypia No atypia	■	■	■
30	B	No atypia	■	■	■
31	B N	No atypia No atypia	■	■	MI
32	B N	No atypia No atypia	■	■	■
33	B	No atypia	■	■	■
34	B	No atypia	■	■	■
35	B N	No atypia No atypia	■	■	■
36	B N	No atypia No atypia	■	■	■
37	B N	No atypia No atypia	■	■	■
38	B N	No atypia No atypia	■	■	■
39	B N	No atypia No atypia	■	■	■
40	B N	No atypia No atypia	■	■	■
41	B N	No atypia No atypia	■	■	■
42	B	No atypia	■	■	■
43	B N	No atypia No atypia	■	■	■
44	B	No atypia	■	■	■
45	B N	No atypia No atypia	■	■	■
46	B N	No atypia No atypia	■	■	■

RESULTS

We tested microsatellite markers for LOH at 3p21, 9p21, and 17p13(TP53) in 80 oral punch biopsies from 46 individuals with a previous history of head and neck or UADT malignancy. All of the biopsied lesions were identified by toluidine blue staining during a large prospective study designed to evaluate the use of OraTest in detecting recurrent or secondary oral cancers (see “Materials and Methods”). Pathological examination of the oral biopsies from 46 patients revealed invasive SCC in 13 cases, CIS and various degrees of dysplasia in 11 cases, and nonneoplastic changes in 22 cases (Table 1).

All of the 80 oral biopsies from 46 patients were examined for LOH at chromosomal arms 3p, 9p, and 17p. Table 1 shows the overall frequency of LOH in individual patients and the distribution of LOH at all loci in the different lesions. LOH was detected in at least one marker in 76% (35 of 46) of all cases. The frequency of LOH was clearly elevated with the increasing pathological severity of the lesions. All 13 (100%) SCC cases, 82% (9 of 11) of CIS or dysplasia, and 59% (13 of 22) of cases with no neoplastic morphology showed LOH in at least one tested marker. Allelic losses at 9p21 were the most frequently detected. LOH at this chromosomal region was detected in 69% (31 of 45) of all cases including 91% (11 of 12) of SCC, 82% (9 of 11) of CIS or dysplasia, and 50% (11 of 22) of nonneoplastic biopsies. Compared with 9p21, LOH was less frequently detected in chromosomal region 3p21 (28%, 9 of 32) and the TP53 locus on 17p13 (32%, 9 of 28) for informative cases.

To determine whether allelic losses at our tested chromosome loci were related to the morphological severity of the secondary lesions, the frequency of LOH at different genetic loci was compared among the different histological groups. No statistical difference was found among the groups for overall frequency of LOH or 9p21 LOH only. However, the frequency of LOH at 3p21 was significantly different between the invasive SCC group (60%, 6 of 10) and the nondysplastic group (14%, 2 of 14; $P = 0.03$). The same significant difference was observed at the TP53 locus (55% in SCC versus 8% in the nonneoplastic group; $P = 0.02$). In addition, the prevalence of LOH at more than one locus was much higher ($P = 0.0001$) in SCC (69%, 9 of 13) than in the nonneoplastic lesions (5%, 1 of 22).

In addition to allelic loss, microsatellite instability was found in four cases (one SCC, two dysplasias, and one nonneoplastic tissue). Although microsatellite instability at these loci does not suggest specific gene inactivation, its presence is a definite marker for clonality (22). One dysplastic lesion (case 15) showed microsatellite instability at two loci (3p21 and 9p21) simultaneously.

Of 46 patients, 34 cases had at least two biopsies collected

Fig. 1 Genetic alterations of 9p21, 3p21, and 17p13(TP53) loci in oral biopsies from patients with prior UADT cancer. Oral biopsies were directed by OraTest (toluidine blue) staining. In the majority of the cases, two punch biopsies were taken from each patient, one from a positive-stained area (B) and another from an adjacent negative-stained area (N). Genetic alterations were tested by microsatellite analysis. ■, retention of the alleles; ■, LOH; □, noninformative or none tested; MI, microsatellite instability; *, LOH present in another 9p21 marker. HDPL, high-grade dysplasia; LDPL, low-grade dysplasia.

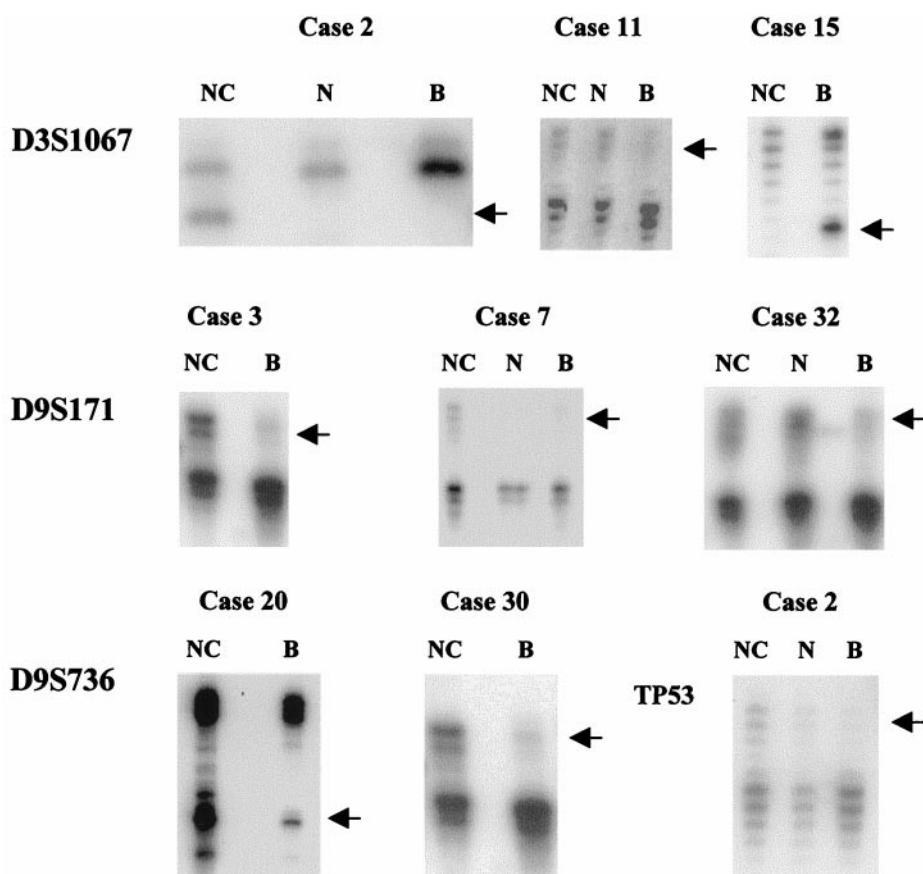


Fig. 2 Representative results of LOH analysis. DNA was isolated from control stroma (Lane NC), OraTest stained area (B) and adjacent nonstained area (N) of each patient. Histopathological morphology of the lesions is indicated in Fig. 1. Arrow, the deleted allele or bandshift (microsatellite instability). Cases 3, 20, and 30 (in which only the stained biopsy was available) showed LOH at *D9S171* and *D9S736*, respectively. In cases 2, 7, 11, and 32, in which two biopsies were analyzed, identical patterns of allelic losses were seen at *D3S1067* and *TP53* (case 2) and at *D9S171* (case 7), whereas LOH involving only the stained biopsy was detected at *D3S1067* (case 11) and *D9S171* (case 32). Case 15 demonstrates a bandshift at *D3S1067*.

from different sites of the oral cavity simultaneously (blue-staining and adjacent nonstaining sites). Twenty-five of 34 cases showed LOH at least in one biopsy at one or more tested markers (Fig. 1). Identical patterns of allelic loss between the two biopsies were found in seven SCC, one dysplastic, and eight nonneoplastic cases regardless of OraTest staining pattern and morphology. Discordant genetic alterations between two biopsies with either the same or a different morphology were detected in four SCC, two dysplasia, and three nonneoplastic cases (Fig. 2). In all but one (case 40) of these 9 cases, the blue-staining region contained LOH at more genetic loci than did the adjacent nonstaining areas.

Chromosome 9p21 was the most frequent identical loss shared between two different biopsies regardless of morphology. Sixty-eight % of cases (17 of 25) had LOH on 9p21 at two adjacent biopsies, whereas only 20% (5 of 25) and 12% (3 of 25) of cases showed identical LOH between two biopsies on 3p21 and the *TP53* locus, respectively. In all five of the LOH-positive SCC cases that had different neoplastic morphology between the two adjacent biopsies, identical LOH involving at least one tested marker on 9p21 was present simultaneously in the invasive SCC and the synchronous dysplastic or phenotypically normal epithelia.

DISCUSSION

Genetic alterations are the hallmark of human cancer. Allelotyping using microsatellite markers has revealed that LOH is

the most frequent genetic changes in a variety of human cancers, including head and neck cancer (10, 12). By virtue of its high frequency and facile detection, LOH has been explored as a useful marker for early detection, chemotherapeutic prevention, and the assessment of further cancer risk (23–25). LOH studies have identified losses on several chromosome arms, especially 3p, 9p21, 11q, and 17p as frequent events in head and neck cancer. Subsequent studies demonstrated a high incidence of LOH on these chromosomal arms in metachronous or synchronous premalignant lesions, suggesting that these genetic alterations are common early alterations in head and neck carcinogenesis (12, 26).

The possibility that LOH can stratify the risk of progression of oral premalignant lesions, was first raised by Mao *et al.* (19) on samples collected during a chemoprevention study. In this prospective study, 37 patients with oral leukoplakia were examined for LOH at 9p21 and 3p14. A striking association between frequency of LOH at these two genetic loci and progression of the leukoplakia to invasive oral cancer was revealed. LOH at 9p21 and 3p14 was found in 37% of progressive cases as compared with only 6% of nonprogressive cases. The notion that losses at either 9p21 and 3p are virtually necessary (occur in close to 100% of progressive lesions) for progression to oral SCC was further confirmed by two recent retrospective studies performed on larger populations (17, 18).

Patients who have had a prior oral or UADT malignancy have a high incidence of second oral cancer. This has been presumably

attributed to the role of "field cancerization" in head and neck carcinogenesis, in which oral and aerodigestive mucosa are continuously exposed to the same environmental carcinogenic insults such as tobacco or alcohol. Fundamental genetic studies in head and neck cancer suggest that a single transformed cell in the oral cavity can spread throughout the mucosa giving rise to large areas of clonally related and transformed cells (20). Additional evidence suggests that, when two lesions arise in one patient, they are often clonal in origin (7). Thus, patients with head and neck cancer often have large patches of abnormal cells that can be directly tested by a simple biopsy.

The OraTest study afforded us the opportunity to explore the issue of whether clonal genetic changes can be identified in suspected lesions that are stained blue by the toluidine dye. We found that LOH in at least one of the tested loci was detected in 76% of oral biopsies from the patients. In the initial multi-institutional OraTest study, investigators identified cancer in only one-third of the 96 biopsied lesions.⁴ Our molecular analysis now definitively shows that three-quarters of the lesions identified by OraTest are in fact clonal. Previous studies from our laboratory suggest that not all preneoplastic lesions will have clonal genetic changes. For example only one-third of hyperplastic lesions had LOH (8) and less than 10% of random biopsies in smokers demonstrated allelic loss.⁵ Moreover, studies from other laboratories suggest that only lesions with clonal genetic changes are likely to progress to cancer (17, 18, 27). This study thus establishes the fact that preneoplastic changes identified by OraTest in this patient population are often clonal and are, therefore, in the progression pathway to cancer. Although it is not certain that everyone of the identified lesions will progress to an invasive cancer, it is clear that the initial clonal expansion has begun in these patients. Accumulating evidence suggests that these clonal patches place these patients in a very high risk category (28).

Chromosome 9p21 remains the most frequently affected locus, and LOH at 9p21 was detected in a substantial proportion (50%, 11 of 22) of phenotypically normal epithelia in addition to SCC and dysplastic lesions. Frequent detection of 9p21 deletion in neoplastic lesions as well as in nonneoplastic oral mucosa confirms that 9p21 loss is an early event in the development of oral malignancy. However, losses of 3p and 17p independently or combined seem to be more predictive of morphological severity and occur only rarely in histologically normal epithelium. Similar results were reported in a recent retrospective study in which premalignant lesions with multiple chromosome losses were found to harbor a 33-fold increase in relative cancer risk as compared with only a 3.8-fold increase when allelic loss at only one chromosome locus was detected (18). Moreover, 17p losses alone occurred in 43–72% of progressing lesions and in only 8–17% of nonprogressing lesions (17, 18). It is thus reasonable to assume that normal appearing biopsies with multiple losses are at very high risk of progression.

Another important point derived from this study relates to the issue of staining and nonstaining areas. In almost all cases, both the staining and nonstaining areas share the same clonal

genetic changes. On the basis of our elucidation of the clonal progression model of head and neck cancer and other studies, it is clear that most patients with head and neck cancer that recur have large patches of abnormal epithelium. It is not surprising that a biopsy adjacent to the staining area would also show the same clonal genetic changes. Importantly, there were three cases in which the biopsy from only the stained region revealed a clonal genetic change not present in the unstained areas. Moreover, another five cases had LOH at more tested loci in the biopsy from the stained area than that from the unstained area. These observations are probably attributable to the presence of smaller clonal patches in some patients and further supports the use of OraTest staining in identifying abnormal lesions.

Clearly, OraTest identified patients with CIS and cancer confirmed by standard morphological analysis. Remarkably, the vast majority of other detected lesions appeared normal or dysplastic under the microscope but still harbored the critical clonal genetic changes that are necessary for cancer progression. OraTest thus represents a powerful method to detect cancers as well as lesions that are likely to progress to cancer. Further stratification of risk with molecular markers thus seems reasonable for biopsied lesions. If confirmed in ongoing prospective studies, these LOH markers may direct additional diagnostic and therapeutic interventions for these patients in the preclinical and preneoplastic phase of oral cancer.

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⁴ Doug Burkett and Joel B. Epstein, personal communication.

⁵ Unpublished data.

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