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# Allelic Imbalance on Chromosome 3p in Oral Dysplastic Lesions: An Early Event in Oral Carcinogenesis[1]

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## Abstract

**We have demonstrated previously a loss of constitutional heterozygosity on the short arm of chromosome 3 in ~50% of oral squamous cell carcinomas. In the present study, we have investigated 30 oral dysplastic lesions (DLs), presenting clinically as either erythroplakias or leukoplakias with histopathological features of either severe epithelial dysplasia or carcinoma *in situ*, for LOH on chromosome 3p using 15 microsatellite markers. Thirteen of the 30 DLs (~43%) showed allelic imbalance at one or more loci. The pattern of loss in these lesions defined three noncontiguous regions of interstitial deletions that overlap with those defined for oral squamous cell carcinomas. These data indicate that the alteration of tumor suppressor genes on chromosome 3p is probably an early event in oral carcinogenesis. Additionally, 7 of the 30 DLs showed microsatellite instability. However, the frequency of loci showing microsatellite instability per lesion was low.**

## Introduction

Approximately 350,000 new cases of oral and oropharyngeal SCCs[3] are diagnosed annually worldwide (1). Two major etiological factors are recognized: tobacco and alcohol. The type and usage of these agents is dependent on social and cultural factors, which to a variable extent underlie the geographic variation in the incidence of oral SCCs. Two major precancerous states are recognized: leukoplakia and erythroplakia. These are defined clinically as white or red patches, respectively, that cannot be attributed to any other disease (2, 3). Overall, the malignant transformation rate of oral precancerous lesions is thought to be between 3 and 6% (2). However, this varies widely with type, site, and degree of epithelial dysplasia (3–5). The early natural history of oral DLs is unclear, and it is difficult to predict the behavior of individual lesions on the basis of current clinical and pathological techniques. LOH on chromosome 3p has been reported in tumors of various tissues, including head and neck carcinomas (Refs. 6 and 7, and references therein). We have demonstrated previously (7) that there are three distinct regions of deletions on chromosome 3p in oral SCCs; this suggests a role for at least three tumor suppressor genes on the short arm of chromosome 3 in oral carcinogenesis. These regions include 3p13–21.1, 3p21.3–23, and 3p25 and overlap with regions of deletion described in other tumors (reviewed in Ref. 7). It has been reported recently that LOH on chromosome 3p occurs at a low frequency in head and neck DLs, suggesting that LOH is a late event in carcinogenesis (8). In the present study, we have investigated for LOH on chromosome 3p specifically in 30 oral DLs using microsatellite markers. We provide evidence that alterations on chromosome 3 may be an early event in oral carcinogenesis.

## Materials and Methods

Genomic DNA was extracted from 30 formalin-fixed, paraffin-embedded oral DLs showing SED or CIS (Table 1) and matching normal tissues as described previously (7). Briefly, all lesions were reassessed by a pathologist to confirm the diagnosis. Lesional and normal tissues were microdissected and subjected to overnight proteinase K [50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.5% Tween 20, with proteinase K at a final concentration of 200 mg/liter] digestion. The digested samples were centrifuged, and the supernatant was used directly for PCR analysis after quantification of the DNA concentration.

Fifteen microsatellite markers mapping to chromosome 3p and two mapping to chromosome 3q were used (Table 2). PCR was carried out using the flanking primers and conditions described previously (9, 10). The amplification reaction was carried out in a final volume of 20 $\mu$l containing 50 ng of genomic DNA, 10 pmol of each primer, 200 $\mu$M of each deoxynucleotide triphosphate, and 0.25 units of Taq polymerase from different suppliers and the reaction buffer supplied with the enzyme (with $MgCl_2$ included at final concentration of 1.5 mM) or a buffer containing 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 15 mM $(NH_4)_2SO_4$, 3.7 mM $MgCl_2$, and 0.85 mg/ml BSA. Amplification products were size fractionated by electrophoresis through 8–10% polyacrylamide nondenaturing gels (Accugel; National Diagnostic) and visualized by silver staining as described previously (7).

Constitutional homozygosity was regarded as uninformative. The signal intensity of the alleles in constitutionally heterozygous samples was analyzed blindly by scanning laser densitometry (Omni Media Scan XRF with Bioimage whole-band analyzer software; Millipore). When the ratio of the two alleles in the lesional tissue differed from that in the matching normal tissue by 30% or more, allele loss was inferred. When the ratio differed by 20% or less, allele retention was inferred. Differences between the ratios of >20% but <30% were regarded as equivocal. MI was scored when new alleles appeared in the lesional tissue compared with the normal tissue. Equivocal allelic imbalance and MI were regarded as uninformative in defining the smallest contiguous regions of loss.

All samples showing allele loss and MI were subjected to repeat analysis after two independent amplifications. In all cases, the replicate test gave identical results.

Clinical details were retrieved from patient records. Fisher's exact probability test, with the accepted significance level of $P < 0.01$ (one-tailed), was used to test for differences in the frequency of allelic imbalance in: (*a*) lesions with and without significant clinical histories (*i.e.*, a previous, concurrent, or subsequent carcinoma, or recurrence of DL, which is highly suggestive of a precancerous lesion); and (*b*) SEDs and CISs.

## Results

**Frequency of Allelic Imbalance on Chromosome 3p.** Thirty oral DLs from 27 unrelated patients were investigated for LOH using 15 microsatellite markers. The clinicopathological details of the DLs are shown in Table 1. The markers and their map positions are shown in Table 2. Specimens OPC5 and -5b, OPC11 and -12, and OPC13 and

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[3] The abbreviations used are: SCC, squamous cell carcinoma; DL, dyplastic lesion; LOH, loss of constitutional heterozygosity; SED, severe epithelial dysplasia; CIS, carcinoma *in situ*; MI, microsatellite instability.

Table 1 *Site of origin, clinical details, and summary of LOH and MI analysis of oral dysplastic lesions*

| No. (OPC-) | Site | Clinical details | Histopathology | LOH[a] | MI[b] |
|---|---|---|---|---|---|
| 1 | Mandible | Concurrent carcinoma | CIS | + | − |
| 2 | Tongue | Previous carcinoma | SED | + | − |
| 3 | Tongue | No recurrence | CIS | − | − |
| 4 | Buccal mucosa | No recurrence | SED | − | − |
| 5 | Floor of mouth | No recurrence | CIS | − | + |
| 5b | Floor of mouth | No recurrence | CIS | + | + |
| 7 | Floor of mouth | Subsequent carcinoma | CIS | + | − |
| 8 | Floor of mouth | No recurrence | CIS | + | − |
| 9 | Tongue | No recurrence | SED | − | − |
| 10 | Buccal mucosa | Subsequent carcinoma | SED | − | − |
| 11 | Floor of mouth | No recurrence | SED | − | + |
| 12 | Floor of mouth | No recurrence | SED/CIS | + | − |
| 13 | Floor of mouth | No recurrence | SED | − | + |
| 14 | Floor of mouth | No recurrence | SED | − | − |
| 15 | Tongue | No recurrence | SED | − | − |
| 16 | Buccal mucosa | Subsequent recurrence | SED | − | − |
| 18 | Buccal mucosa | Previous carcinoma Subsequent carcinoma | SED | + | − |
| 19 | Buccal mucosa | Subsequent carcinoma | SED | − | − |
| 20 | Palate | No recurrence | SED | − | − |
| 21 | Buccal mucosa | No recurrence | SED | − | − |
| 22 | Tongue | Previous carcinoma | CIS | − | − |
| 23 | Tongue | Subsequent recurrence | SED | + | − |
| 24 | Tongue | Previous carcinoma | SED | − | + |
| 25 | Floor of mouth | Previous carcinoma | CIS | + | + |
| 26 | Tongue | Previous carcinoma | SED | + | − |
| 27 | Tongue | Subsequent recurrence | SED | + | + |
| 29 | Tongue | No recurrence | SED | + | − |
| 30 | Tongue Floor of mouth | Previous dysplastic lesion | SED | − | − |
| 31 | Tongue | Previous dysplastic lesion | SED | + | − |
| 34 | Tongue | No recurrence | SED | − | − |

[a] +/−, presence/absence of LOH at one or more loci.
[b] +/−, presence/absence of MI at one or more loci.

-14 were derived from the same lesions from three patients: OPC5, OPC11, and OPC13 were incisional biopsies; OPC5b, OPC12, and OPC14 were excision biopsies of these lesions done within 6 months of the first biopsy.

All lesions proved to be constitutionally heterozygous for at least nine markers, and each marker was informative in at least 11 lesions. Because of a lack of material, a maximum of three markers were not scored for some lesions, and a maximum of eight samples were not examined with one marker (*D3S1228*).

Thirteen of the 30 samples (~43%) showed allelic imbalance at one or more loci on chromosome 3p. The data are summarized in Table 1 and Fig. 1, and representative gels are shown in Fig. 2*A*. Allelic imbalance was observed in three noncontiguous regions in one sample, two noncontiguous regions in nine samples, and a single region in three samples. The presence of interstitial losses excluded the possibility of chromosomal imbalance (*i.e.*, deletion of the whole chromosome accompanied by the duplication of the homologue).

Table 2 *Chromosome 3 microsatellite markers used for LOH analysis*

| Locus (*D3S-*) | Map position[a] |
|---|---|
| *1038* | 3p25 |
| *656* | 3p25 |
| *1110* | 3p25 |
| *1111* | 3p25 |
| *647* | 3p23 |
| *1007* | 3p21.3–22 |
| *966* | 3p21.3 |
| *1029* | 3p21.2–21.3 |
| *643* | 3p21.3 |
| *663* | 3p21.3 |
| *1076* | 3p21.1 |
| *1067* | 3p14.1–21.1 |
| *1228* | 3p14.1–14.3 |
| *659* | 3p13 |
| *1079* | 3p13 |
| *1271* | 3 cent–q13 |
| *1262* | 3q27 |

[a] The map positions are determined from various sources (Ref. 7 and references therein).

**Mapping of the Noncontiguous Regions of Allelic Imbalance.** The most proximal region of allelic imbalance maps between *D3S1079* (3p13) and *D3S1067* (3p14.1–21.1) and centers on the two intervening loci (*D3S659* and *D3S1228*). Overall, 10 of the 26 DLs (~38%) informative at either *D3S659* or *D3S1228* showed allelic imbalance at either of the two loci (Fig. 1). The next region of imbalance maps between *D3S643* (3p21.3) and *D3S1111* (3p25). Nine of the 30 DLs (~30%) informative at one or more of the four intervening loci showed allelic imbalance (Fig. 1). However, of these, only one DL (OPC2) showed a deletion that was not contiguous with either the proximal or the distal regions of deletion. The most telomeric region maps between *D3S1111* (3p25) and *D3S1038* (3p25). Eleven of the 28 DLs (~39%) informative at either of the two intervening loci showed allelic imbalance (Fig. 1).

**Microsatellite Instability.** Seven DLs showed evidence of MI. Of these, one showed instability at one locus, five showed instability at two loci, and one showed instability at three loci. Representative gels are shown in Fig. 2*B*. Three of the seven DLs (OPC5b, OPC25, and OPC27) also showed allelic imbalance on chromosome 3p.

**Differences in Allelic Imbalance and MI in Specimens from Metachronous Biopsies of the Same DLs.** Two of the three DLs (OPC5b and OPC12) showed allelic imbalance in the tissues derived from a second biopsy but not from the first biopsy of the same lesion (OPC5 and OPC11). The reverse situation was observed with respect to MI; OPC14 (second biopsy) failed to show MI, whereas the tissues from the preceding biopsy (OPC13) showed MI at three loci. MI was observed at one locus (*D3S966*) in both OPC5 and OPC5b; however, OPC5 but not OPC5b showed MI at another locus (*D3S1067*). The loci showing MI (*D3S1067* and *D3S966*) in OPC11 showed allelic imbalance in the second biopsy of the same DL.

| | 3 regions[a] | 2 regions[a] | | | | | | | | 1 region[a] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LOCUS (D3S-)[b] | OPC2 | OPC1 | OPC5b | OPC8 | OPC12 | OPC18 | OPC23 | OPC27 | OPC29 | 0PC31 | OPC7 | OPC25 | OPC26 | |
| **1038** | ● | ○ | U | ● | U | ○ | ● | ○ | ● | U | ○ | ○ | ○ | |
| **656** | ● | U | ● | ● | U | ○ | ● | ● | ● | ● | U | ○ | ● | |
| **1110** | ● | ● | U | U | ● | ● | U | U | ● | ● | U | ○ | U | |
| **1111** | ○ | ● | U | U | U | E | ● | U | ○ | U | U | U | ● | |
| **647** | ● | ● | ● | ● | ● | U | ● | ● | U | ● | ○ | ○ | ○ | |
| **1007** | U | ○ | U | U | U | U | U | U | U | E | U | ○ | U | |
| **966** | ● | E | MI | ○ | ● | U | ● | MI | U | ○ | ○ | MI | U | |
| **1029** | ● | U | U | ○ | ○ | ● | ○ | ○ | ○ | ○ | ND | ○ | ○ | |
| **643** | ○ | U | ○ | ○ | ● | ● | ● | ● | ● | ○ | ○ | ○ | ○ | |
| **663** | E | ○ | ○ | ○ | ● | ○ | ● | MI | ● | ○ | ND | MI | U | |
| **1076** | ● | E | ○ | ● | ● | U | ● | ● | ● | ○ | ○ | ○ | ○ | |
| **1067** | ● | U | ○ | ● | ● | ● | ● | ● | ● | ● | ○ | ○ | U | |
| **1228** | ● | U | ● | U | ● | ● | ● | ● | U | ND | ND | U | U | |
| **659** | ● | ● | ● | ● | U | ● | ● | ● | U | ● | U | ● | ○ | |
| **1079** | ● | ○ | ● | U | ● | U | U | U | E | E | ● | ● | ○ | |

Fig. 1. Details of LOH analysis of the 13 oral DLs (OPC-) showing allele loss on chromosome 3p. *a*, lesions are subdivided according to the number of noncontiguous regions of deletions. *b*, the loci appear in descending order from chromosome 3p telomere (*top*) to chromosome 3 centromere. The map positions of the loci are given in Table 2. ●, LOH; ○, retention of heterozygosity; *U*, uninformative; *MI*, microsatellite instability; *E*, equivocal; *ND*, not done. The *solid blocks* on the *right* indicate the smallest common regions of deletion.

**Correlation of Allelic Imbalance with Clinical and Histopathological Features of Oral DLs.** The difference between the frequency of allelic imbalance in DLs with a significant clinical history and those without significant clinical history was not statistically significant (Fisher's exact probability test; $P > 0.05$). Similarly, there was no significant difference between the frequency of allelic imbalance in DLs graded histopathologically as SEDs and those graded as CISs (Fisher's exact probability test; $P > 0.05$).

## Discussion

We have shown previously that LOH on chromosome 3p occurs relatively frequently in oral SCCs (7). In the present study, we have found that allelic imbalance on chromosome 3p occurs only slightly less commonly in oral DLs, which suggests that these alterations may represent early events in oral carcinogenesis. A much lower frequency of LOH on chromosome 3p in head and neck DLs has been reported recently (8). That study also showed a much lower frequency of LOH on chromosome 9 in oral DLs than was shown in a previous study (11). The differences in our study may be due to several factors: (*a*) unlike the previous study (8), we used paraffin-embedded material instead of frozen tissues, which has allowed us to grade the level of dysplasia more accurately; (*b*) we used 15 markers instead of 3 to test for allele loss; and (*c*) excessively high thresholds may have been used in reporting allele loss in the previous study (8). There is unavoidable contamination of the microdissected lesional tissue by connective tissue papillae and lymphocytic infiltrate. Moreover, DLs may consist of polyclonal populations since the present and previous studies (12) have shown that analysis of different biopsies of the same lesion may reveal differences in LOH and MI. Thus, the use of excessively high thresholds will reduce the sensitivity of the analysis. Our criteria for scoring allele loss are similar to those used in other studies (13, 14). We have relied on detecting consistent and reproducible differences by densitometry because we[4] and others (15) have shown that in lesions with >20% contamination by normal tissues, LOH may not be detectable by visual assessment. The nonrandom LOH and overlap of the regions of allelic imbalance in the present study with those defined previously in oral SCCs by us (6) and others (7) provide additional support for the reliability of our method.

Three distinct regions of deletions were identified in oral DLs: 3p13–21.1, 3p21.3–25, and 3p25. These overlap with those defined by us and others in head and neck (including oral) SCCs (6, 7) but do not refine the existing deletion maps. An interesting difference was observed in the frequencies of deletions in each of these regions in oral DLs as compared with oral SCCs. Whereas in the oral DLs the frequencies of loss in the most proximal region (38%) and the telomeric (39%) region were similar to that observed in oral SCCs (44% for both regions; Ref. 7), the frequency of loss in the central region (30%) was lower than that in oral SCCs (50%; Ref. 7). Approximately one-half of the informative cases of head and neck (including oral) SCCs show loss at *D3S1007*, which maps to this region (6, 7). Of the 10 informative DLs in the present study, none showed LOH at this locus. These data appear to suggest that molecular alterations at the most proximal region and the telomeric region may represent early changes, whereas the changes in the central region occur at a later stage. However, only one lesion (OPC2) had a deletion that was not contiguous with either the proximal or the distal region of deletion. Thus, the deletions in the other DLs may involve gene(s) in the central region.

We failed to demonstrate any statistically significant difference in the frequency of allelic imbalance between DLs that had been graded as SED and those that had been graded as CIS. This is probably a reflection of the subjective nature of the histopathological diagnosis

[4] Unpublished data.

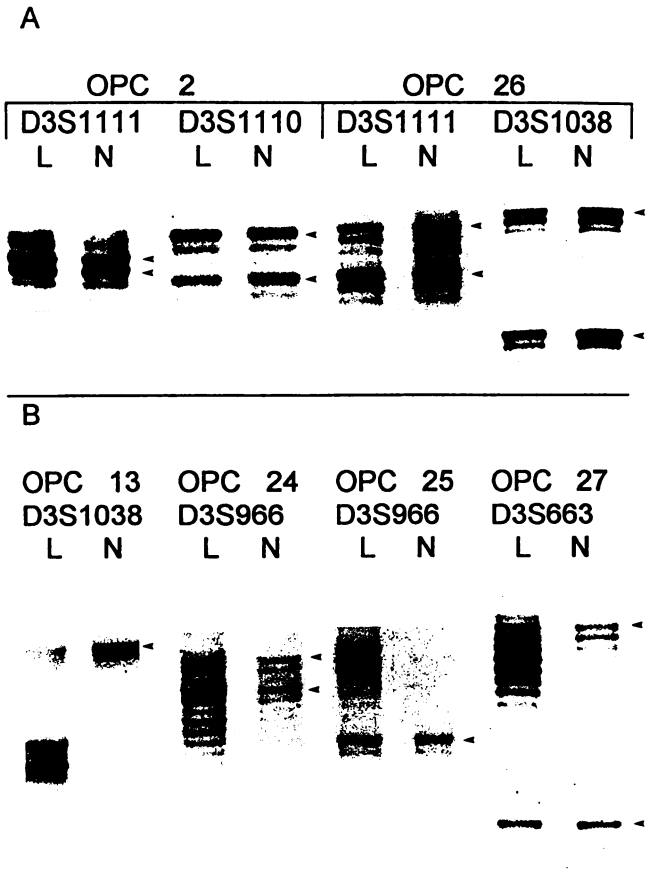


Fig. 2. Silver-stained polyacrylamide gels showing products of PCR-amplified chromosome 3p microsatellites from matched lesional (*L*) and normal (*N*) DNA. The loci and sample numbers are indicated above the gel lanes. *A*, LOH analysis in oral DLs. *Arrowheads*, positions of the alleles. In some cases, in addition to the main allele(s), faster-migrating stutter bands and more slowly-migrating conformational bands are present. *OPC2* shows loss of the lower allele at the *D3S1110* locus and retention of heterozygosity at the *D3S1111* locus. *OPC26* shows loss of upper allele at the *D3S1111* locus and retention of heterozygosity at the *D3S1038* locus. *B*, MI in oral DLs. *Arrowheads*, normal alleles. In each case illustrated, the lesion shows reproducible additional bands. These represent altered alleles resulting from either expansion or contraction of the normal allele(s).

rather than a reflection of the chronology of events in oral carcinogenesis. There were also no statistically significant differences between DLs with significant clinical histories (*i.e.*, previous, concurrent, or subsequent carcinoma, or recurrence of DL) and those without a significant history. However, such analysis does not allow for high-risk activities, such as tobacco and alcohol use, by individual patients and the variability in the period of follow-up of individual lesions.

We observed MI in 7 of the 30 DLs. However, the frequency of loci showing MI per lesion was low. Thus, MI is unlikely to be indicative of a replication error-positive phenotype. The mechanisms involved in such low-frequency DL/tumor-specific MI are unclear.

A surprising feature of our findings was the discrepancy in allelic imbalance and MI between different specimens from the same DL. This may be due to the presence of heterogeneous polyclonal populations in the DLs. Variations in genetic alterations between different areas of the same lesion have also been reported recently in ductal CIS of the breast (12). The differences could also be due to progression of the DLs. However, although the biopsies were metachronous, in each case, there was a difference of <6 months. The differences in allelic imbalance may also reflect the level of contamination by normal tissues in the different samples. Depending on the size of the DLs, one of the pair of biopsies of the same lesion had a majority of the lesional tissue, while the other had smaller areas of such tissue. The smaller biopsies were generally more difficult to microdissect.

The present study, therefore, demonstrates that LOH on chromosome 3p is found in oral DLs at a relatively high frequency and is probably an early event in oral carcinogenesis. Larger retrospective studies of oral DLs displaying all grades of dysplasias and with known outcomes are necessary to determine: (*a*) at what stage in oral mucosal carcinogenesis alterations on chromosome 3p occur; and (*b*) the value of such alterations in predicting malignant transformation. Additional studies are also necessary to determine the level of heterogeneity in such changes in different areas of the oral DLs.

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