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Allelic imbalance in oral lichen planus and assessment of its classification as a premalignant condition

Brent T. Accurso, DDS, MPH^a, Blake M. Warner, MPH^b, Thomas J. Knobloch, PhD^c, Christopher M. Weghorst, PhD^c, Brian S. Shumway, DDS, MS^d, Carl M. Allen, DDS, MSD^a, and John R. Kalmar, DMD, PhD^a

THE OHIO STATE UNIVERSITY COLLEGE OF DENTISTRY, COLLEGE OF PUBLIC HEALTH, AND COMPREHENSIVE CANCER CENTER, AND UNIVERSITY OF LOUISVILLE

Abstract

OLP is a relatively common immune-mediated mucosal condition with a predilection for middle-aged women. Although classified as a premalignant condition, this classification remains controversial. Using stringent diagnostic criteria, some authors have found that OLP patients are not at increased risk for oral SCC. Credible but limited genetic evidence also indicates that epithelial tissues from OLP patients diagnosed using stringent criteria differs from premalignant or malignant oral lesions but is similar to epithelium from benign oral lesions. To further investigate this genetic line of evidence, biopsy specimens diagnosed as fibroma, OLP, low-grade dysplasia, high-grade dysplasia, and SCC were retrieved from the archives of the Oral Pathology Consultants at the Ohio State University. Using laser capture microdissection, tissue of interest was captured from each case and DNA subsequently extracted. Fluorescently labeled PCR primers were used to amplify DNA at 3 tumor suppressor gene loci (3p14.2, 9p21, and 17p13) and evaluated for LOH or microsatellite instability (MSI). OLP was found to be significantly different from low-grade dysplasia, high-grade dysplasia, and SCC when LOH/MSI was found at more than 1 loci ($P = .011$, $P = .032$, $P = .003$), but not different from benign fibromas ($P = .395$). In agreement with previous studies, well-documented cases of OLP diagnosed using stringent criteria exhibit a genetic profile more similar to a benign or reactive process than a premalignant/malignant one. These findings do not support the classification of OLP as a premalignant condition.

Lichen planus is a common immune-mediated disease that can affect the skin, mucous membranes, or both.¹ It is most common in adult women with an age-standardized prevalence of 1.27% (0.96% in men, 1.57% in women).² The prevalence of lichen planus in the oral cavity has been estimated to be between 0.1% and 2.2%.¹ On the skin, lichen planus has classically been described as purple, polygonal, pruritic papules that may exhibit fine, interlacing white lines known as Wickham striae.³ In the oral cavity, lichen planus typically presents in a papuloreticular pattern with striae located on the posterior buccal mucosa bilaterally, although it can occur on other mucosal surfaces, such as the lips, tongue, and gingiva.¹ In most cases, the mucosal changes of lichen planus are asymptomatic and the patient may be unaware of their presence; in other patients, the lesions can become erosive or ulcerated and quite painful.

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Reprint requests: Brent T. Accurso, DDS, MPH, The Ohio State University College of Dentistry, Oral and Maxillofacial Pathology, 305 West 12th Avenue, Columbus, OH 43210, brent.accurso@gmail.com.

^aThe Ohio State University College of Dentistry.

^bThe Ohio State University College of Public Health.

^cThe Ohio State University Comprehensive Cancer Center.

^dUniversity of Louisville, Department of Surgery and Hospital Dentistry.

The histopathologic changes of oral lichen planus (OLP) are characteristic and classically consist of atrophy of the surface epithelium with hyperkeratosis, absent or saw-toothed rete ridges, and a bandlike infiltrate of lymphocytes immediately subjacent to the basement membrane with associated destruction of the basal layer.^{1,3} This “lichenoid” pattern, however, can be seen in other reactive or immune-mediated processes, such as contact reactions, graft-versus-host disease, and lupus erythematosus.³ Additionally, it should also be considered that a bandlike pattern of inflammation can be seen as a response to overlying dysplastic surface epithelium and mimic the histopathologic features of OLP.^{4,5}

Most experts agree that lichen planus is an autoimmune disease primarily related to T-lymphocyte dysfunction that induces apoptosis in basal keratinocytes.^{3,6} What triggers the T-lymphocyte attack on the surface epithelial cells remains unknown. Hypotheses have included an antigen-specific cell-mediated immune response, nonspecific responses to the basement membrane, a true autoimmunity, including loss of immune privilege by basal keratinocytes, or a humoral immunity owing to reports of circulating autoantibodies in OLP patients.⁶ The antigen-specific response hypothesis implicates a variety of agents, such as systemic drugs, dental materials, trauma, or microbial organisms.⁶ The role of hepatitis C virus in the development of lichen planus is an area of active investigation, with some theorizing that expression of viral antigens on the surface of the basal keratinocytes triggers a response by CD8⁺ T lymphocytes.⁷ A recent meta-analysis found an association between hepatitis C virus infection and lichen planus, but this association was limited to certain geographic regions (East and Southeast Asia, South America, Middle East, and Europe).⁷ These authors suggested that screening patients with lichen planus for occult hepatitis C infection may be warranted.⁷

The suggestion that OLP predisposes affected patients to develop oral SCC has been debated for decades. Premalignant oral epithelium is typically defined by histopathologic criteria as different grades of epithelial dysplasia depending on the severity of the microscopic changes.¹ Dysplastic epithelium is considered at increased risk for transformation into squamous cell carcinoma (SCC), with high grades transforming at a greater frequency.¹ The cutaneous version of lichen planus is acknowledged to be benign, but many authorities, including the World Health Organization, classify OLP as a premalignant condition.^{2,3} Cases of purported malignant transformation of OLP have appeared in the literature for more than 100 years.⁸ More recently, however, several authors have suggested that the vast majority of such reports provided insufficient documentation for a diagnosis of OLP and advocated more stringent histopathologic and clinical criteria.^{9–11} Others have postulated that some purported cases of malignant transformation of OLP were actually cases of dysplastic oral epithelium with a concomitant lichenoid reaction.^{3,4} Additionally, a known premalignant condition with a high transformation rate, proliferative verrucous leukoplakia (PVL), can bear a striking, and potentially confusing, clinical and microscopic resemblance to OLP.¹²

Recent prospective cohort studies by van der Meij et al.^{8,13} further described the epidemiology of potential cancer development in OLP. This study used stringent histopathologic and clinical diagnostic criteria for OLP; oral lesions meeting some, but not all, of the criteria were classified as oral lichenoid lesion (OLL) and not OLP. Of the 192 patients in the updated study, 4 developed SCC during the follow-up period and each of these 4 cases occurred in patients with OLL.¹³ The authors calculated the malignant transformation rate of OLL to be 0.71% per year and concluded that patients with OLL, but not OLP, should be considered at risk for the development of oral SCC and warrant close clinical follow-up.¹³

In addition to clinical and histopathologic studies, other authors have focused on the genetic characteristics of OLP lesions compared with oral premalignant and malignant lesions. A study by Zhang et al.⁵ evaluated loss of heterozygosity (LOH) at tumor suppressor gene loci commonly altered in oral SCC, comparing cases of OLP, dysplastic oral epithelium, and oral SCC. The authors found that LOH was an infrequent finding in OLP and occurred at a far higher frequency in cases of oral epithelial dysplasia and oral SCC. Increasing percentages of LOH were found in association with increasing degrees of dysplasia and the highest amount of LOH was seen in cases of SCC. Furthermore, the presence of LOH at more than 1 locus was significantly associated with dysplasia and SCC. Loss of genetic information at multiple arms has been demonstrated by others to be a significant predictor in the progression of premalignant oral epithelial lesions to SCC.^{14,15} This genetic evidence, in conjunction with the epidemiologic findings of van der Meij and others,¹³ supports the concept that OLP is not a premalignant condition. We concur with the hypothesis that OLP, when diagnosed using stringent clinical and histopathologic criteria, is more similar to benign oral epithelium than premalignant or malignant oral epithelium with respect to genetic changes at key tumor suppressor genes. The intent of this study was to confirm this hypothesis and the findings of Zhang et al.⁵ through analysis of tumor suppressor gene loci using DNA isolated from selected archived, paraffin-embedded tissue specimens.

METHODS

Selection of cases for analysis

Following approval from the Biomedical Institutional Review Board at the Ohio State University (protocol # 2010H0084), the archives of the Oral Pathology Consultants at the Ohio State University College of Dentistry were searched for specimens diagnosed within the previous calendar year (2009) as fibroma (normal control); lichenoid mucositis; mild, moderate, or severe epithelial dysplasia; carcinoma in situ, or SCC. Cases of mild and moderate epithelial dysplasia were combined and renamed “low-grade epithelial dysplasia” and the cases of severe epithelial dysplasia and carcinoma in situ were combined and renamed “high-grade epithelial dysplasia” for a total of 5 groups. All cases of lichenoid mucositis were limited to cases in which the clinical information (documented through photos, drawings, or detailed written descriptions) fit the clinical criteria of OLP. Specifically, a bilateral and symmetric distribution of reticular striae on the oral mucosa was necessary to be clinically consistent with OLP.¹³ After viewing the archived hematoxylin-eosin-stained slides to confirm the diagnosis, 10 formalin-fixed, paraffin-embedded tissue blocks were retrieved for each of the 5 groups for this study. Of the 10 cases of lichenoid mucositis, 7 were described as “erosive” by the clinician. All 7 cases of erosive OLP were simultaneously submitted in Michel’s solution for direct immunofluorescent studies to exclude more specific, antibody-mediated vesiculo-erosive conditions.

Laser capture microdissection and DNA isolation

Polyethylene naphthalate (PEN)-coated membrane slides (Carl Zeiss MicroImaging, LLC, Thornwood, IL) were pretreated with ultraviolet light for 30 minutes in a Stratalinker UV Crosslinker (1800) (Stratagene, La Jolla, CA). Tissue sections (8 µm) were cut from the blocks, placed on the slides, and subsequently air dried overnight in a dessicator. The slides were heated in an oven at 95°C for 10 minutes to melt the paraffin, deparaffinized in the standard fashion with xylene, the xylene was cleared using descending alcohol washes, and the slides were stained using hematoxylin-eosin. Laser capture microdissection was performed at the Laser Capture Molecular Core of The Ohio State University Medical Center using a PALM MicroBeam IV laser microdissection and pressure-catapulting system (Carl Zeiss Group, Bernried, Germany). Two samples, consisting of lesional epithelium and underlying normal stromal tissue, were microdissected from each case. The amount of tissue

captured for each sample ranged from 500,000 to 1 million μm^2 in total area. Examples of the tissue, before and after laser capture, are displayed in Fig. 1. DNA was extracted from each sample using proteinase K digestion and a QIAamp DNA Micro Kit (Qiagen, Valencia, CA) per the manufacturer's instructions.

Polymerase chain reaction amplification

DNA was amplified by 5'-fluorescently labeled custom polymerase chain reaction (PCR) primers (Applied Biosystems, Foster City, CA). The 3 microsatellite markers used in this study were selected from sites previously used to evaluate presumed tumor suppressor loci in oral SCC.^{5,16} The selected markers were located at 3p14.2 (*D3S1300*), 9p21 (*D9S1748*), and 17p13 (*TP53*) and were amplified using the fluorescently labeled primer pairs listed in Table I.

A 20- μL PCR reaction was performed, consisting of 10 μL of 2 $\times$ TaqMan Gene Expression Master Mix (Applied Biosystems), 5 μL of genomic DNA (gDNA), 1 μL of custom primer set, and 4 μL of water. In some cases, the PCR product was insufficient for accurate microsatellite analysis; these PCR reactions were repeated with increased gDNA content (up to 10 μL). The DNA was amplified using an Applied Biosystems Veriti 96-well thermocycler under the following reaction conditions: 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, 59°C for 45 seconds, 68°C for 1 minute, and a final elongation step at 68°C for 7 minutes.

Loss of heterozygosity/microsatellite instability analysis

The LOH analysis was performed at the Plant Microbe Genomics Facility at the Ohio State University using an Applied Biosystems 3730 sequence analyzer to separate and visualize fluorescently labeled PCR products via capillary electrophoresis. GeneScan 600 LIZ (Applied Biosystems) served as the size standard. Peaks were analyzed using GeneMapper v4.0 and Peak Scanner v1.0 software (Applied Biosystems).

LOH determinations were made by first calculating 2 ratios: one ratio of the peak areas for the epithelial tissues of interest and another for the peak areas of the underlying normal stromal tissue.^{17,18} These ratios were then divided to make a ratio of ratios, with LOH defined if this ratio of ratios was less than 0.6 or greater than 1.67. Peak intensities that were 100 or fewer relative fluorescent units were excluded for being within background. Microsatellite instability (MSI) was defined as mobility of the allele, usually seen as several additional peaks.¹⁷⁻¹⁹ LOH and MSI were grouped together for statistical analysis and categorized as AI. In several instances, the PCR products did not sufficiently amplify to allow for analysis and were deemed not informative, or NI. Samples were assigned 1 classification and scored as being normal, NI, or AI. Examples of normal, MSI, and LOH are displayed in Fig. 2.

Statistical analysis

Fisher's exact test was chosen to compare the allelic changes among the different groups using JMP statistical software (SAS; Cary, NC). One-way analysis of variance (ANOVA) was used to check for differences between the demographic distributions of the groups. Results with a *P* value of .05 or less were considered significant.

RESULTS

Demographic analysis

As presented in Table II, the specimens in this study came from patients in middle-to-late age (mean: 59.9 SD: 12.9, range: 22–90) without significant difference with respect to age

($P = .207$). The patients were predominantly female (60%) but this difference was not significant ($P = .395$). Unfortunately, the patient histories provided in the selected cases were not consistently detailed with regard to well-known risk factors for oral cancer (e.g., smoking, alcohol use) to allow for analysis of these risk factors. The total number of informative cases in the groups ranged from 22 to 30 and were not significantly different between groups ($P = .161$) or by marker ($P = .242$) (Table II).

Allelic imbalance at any marker

AI at any single marker was found to occur at a lower frequency in the fibroma and OLP groups (60% and 50% of cases, respectively) compared with the low-grade dysplasia, high-grade dysplasia, and SCC groups (90%, 90%, and 100% of cases, respectively). Using Fisher's exact test (Table III), AI at any marker was found to be significantly different only between the fibroma group and the SCC group ($P = .043$) and the OLP group and the SCC group ($P = .016$). Although not statistically significant, AI at any marker approached significance when comparing the OLP group and low-grade or high-grade dysplasia groups ($P = .065$). The differences between all other groups were not found to be statistically significant.

Allelic imbalance at more than 1 marker

Allelic imbalance at multiple arms has been shown to be more relevant than loss at one site.^{5,14,15} The frequency of AI at more than 1 marker in the fibroma and OLP groups (10% and 20% of cases, respectively) was notably smaller than in the low-grade dysplasia, high-grade dysplasia, and SCC groups (80%, 70%, and 90% of cases, respectively). Comparisons between groups using Fisher's exact test are presented in Table IV. They demonstrate that the fibroma and OLP groups are not different from each other ($P = .395$), but both are significantly different from low-grade dysplasia (fibroma: $P = .003$; OLP: $P = .011$), high-grade dysplasia (fibroma: $P = .009$; OLP: $P = .032$), and SCC (fibroma: $P < .001$; OLP: $P = .003$). Low-grade and high-grade dysplasia were not different from each other ($P = .348$), nor were they different from SCC ($P = .395$, $P = .2$). Tables V–VII present the results of Fisher's exact test for AI by marker. Interestingly, these results indicate that most of the statistical association for differences between the AI of each group is derived from the *TP53* marker (Table VII).

DISCUSSION

OLP remains a highly controversial condition with regard to its status as a premalignant condition. Some authorities have argued that an accurate diagnosis can be made only using both histopathologic and clinical criteria and that many ostensible cases of malignant transformation of OLP fail to meet these criteria.^{9–11} Van der Meij and colleagues¹³ posited that in the absence of both criteria, the term “oral lichenoid lesion” should be used and demonstrated in their study that malignant transformation occurred only in OLL, not in accurately diagnosed cases of OLP. Additionally, the premalignant condition known as PVL can bear a striking resemblance in its early phase to OLP.^{11,20} As PVL was not described until 1985,²⁰ cases of malignant transformation of OLP reported prior to 1985 may have been PVL, inadvertently strengthening the concept of OLP as a premalignant condition. Despite the advocacy of many to apply stringent criteria when diagnosing OLP,^{4,13} even recent reports of malignant transformation of OLP do not follow these criteria.^{21,22} Additionally, controversy also exists because some have speculated that erosive OLP is at greater risk for malignant transformation.^{8,23} Although not specifically considered during the selection of cases, retrospective examination demonstrated that 7 of the 10 OLP cases were described as “erosive” by the clinician. All 7 had been submitted for direct immunofluorescent studies to rule out other vesiculo-erosive conditions. Chronic ulcerative

stomatitis (CUS), in particular, can mimic OLP at both the clinical and histopathologic levels.²⁴ A diagnosis of CUS is made by immunofluorescence studies to detect autoantibodies to Δ Np63 α , a p63 isoform and normal component of stratified squamous epithelium.²⁴ The p63 protein functions in epithelial proliferation,²⁴ and it could be hypothesized that autoantibodies disrupt this normal function and aid in the development of oral SCC.

In addition to epidemiologic evidence, genetic evidence provides important insight into the true nature of OLP. In this study, the aim was to corroborate the findings of Zhang et al.,⁵ which demonstrated that LOH at tumor suppressor gene loci was far more common in dysplastic and malignant oral epithelium than in OLP; OLP was demonstrated to be more similar to benign oral epithelium. Earlier studies established that LOH most frequently occurred in dysplastic oral lesions and in oral SCC at 3p, 9p, and 17p.^{14,25–27} Because of the increased presence of LOH at these sites in dysplastic lesions, loss of genetic material at these sites should be considered an important step in the development of oral SCC.²⁶ Allelic imbalance, whether LOH or MSI, is believed to contribute to carcinogenesis by inactivating 1 of the 2 alleles of tumor suppressor genes.^{16,18} Complete loss of this tumor suppressor function by disruption of the remaining normal allele by various means would allow for the eventual transformation into a malignancy. Recently published epidemiologic studies have improved our understanding in that area, but no studies have investigated the genetic features of OLP in a similar manner.

The results of this study (Table II) demonstrated that AI, defined as LOH or MSI, occurred at higher rates in epithelial dysplasia and SCC than in OLP or fibromas. When these results were extended to evaluate AI at more than 1 locus, OLP and fibromas were not found to be different from each other ($P = .395$), but were quite different from dysplasias or cancers (all P values $< .05$). A number of studies have found that loss at multiple sites is significantly associated with dysplasias and cancers compared with benign lesions.^{5,14,15,28} One study found an almost 4-fold increase in the risk of progression to SCC in cases with LOH at either 3p or 9p, but a 33-fold increase in cases that had LOH at an additional site.¹⁵ Loss at 3p and/or 9p has been shown to be an early event in the development of SCC.^{14,25,27} In this study, all 10 cases of SCC demonstrated AI at either 3p or 9p, with 6 demonstrating AI at both. In the cases of dysplasia, 80% demonstrated AI at either 3p or 9p, and 40% at both loci. The OLP group demonstrated AI at 3p or 9p in 5 cases, 2 of which had it at both sites and the OLP group was significantly different from both SCC and the dysplasia groups (all P values $\leq .05$).

Of particular note (Tables V–VII) is the contribution of AI at the *TP53* (17p) marker in distinguishing between premalignant/malignant cases and benign cases compared with *D3S1300* (3p) and *D9S1748* (9p). The *TP53* marker corresponds to the TP53 tumor suppressor protein, a key regulator in cell cycle inhibition and/or apoptosis following DNA damage.²⁹ AI at 17p (*TP53*) occurred in low-grade dysplasia, high-grade dysplasia, and SCC (70%, 80%, 90%, respectively) at a far higher frequency than in fibromas or OLP (0%, 10%, respectively).

In this study, AI at 3p was a marginal contributor to detecting differences between the groups, and AI at 9p was not a useful discriminator. Although these results are different from other published studies,^{5,14,15,25,26} given the smaller sample size in this study, the larger number of markers and the consistency of results in the aforementioned studies, our results should be viewed with caution and no inferences should be made regarding AI at 9p. In a number of cases, the PCR product was not initially interpretable, thereby yielding a noninformative case. These samples were subsequently repeated with an increased number of PCR cycles to yield sufficient product for analysis. Most of these cases occurred with the

connective tissue control for the *D3S1300* marker in the fibroma group. Because the connective tissue controls of the fibroma samples are mostly collagen and lack ubiquitous nuclei, and the size of the PCR gene product is larger than the other markers, it is possible that the *DS1300* primer was not able to amplify a sufficient amount of gene product for LOH evaluation.

In conclusion, the results of this study are in agreement with the results of an earlier study.⁵ When diagnosed using stringent histopathologic and clinical criteria that exclude potential mimics, particularly PVL and CUS, OLP is more like benign oral tissue with regard to allelic imbalance at 3 key tumor suppressor genes than premalignant or malignant oral tissue.

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Fig. 1.
Examples of OLP (**A**), low-grade dysplasia (**B**), and SCC (**C**) before and after laser capture (hematoxylin-eosin stain; magnification $\times 10$).

Fig. 2.

Examples of (A) electropherogram demonstrating a normal pattern (OLP case # 15, marker *TP53*), (B) electropherogram demonstrating MSI pattern (SCC # 12, marker *D9S1748*), and (C) electropherogram demonstrating LOH pattern (SCC case # 11, marker *D3S1300*). Note: x-axis values denote size in base pairs; y-axis denotes fluorescence intensity.

Table I

Forward and reverse primer sequences and expected product size

Gene locus (marker)	Forward primer	Reverse primer	Expected product size (base pairs)
3p (<i>D3S1300</i>)	5'-AGCTCACATTCTAGTCAGCCT	5'-GCCAATCCCCAGATG	217–241
9p (<i>D9S1748</i>)	5'-CACCTCAGAAGTCAGTGAGT	5'-GTGCTTGAAATACACCTTTCC	130–150
17p (<i>TP53</i>)	5'-GGGATACTATTCAGCCCGAGGTG	5'-ACTGCCACTCCTTGCCCCATTC	103–135

Table II

Results and patient demographics by group

Diagnosis	Marker	Informative cases (P = .161)*	Cases with MSI (%)	Cases with LOH (%)	Total AI (%)	AI at any marker (%)	AI at >1 marker (%)	Mean age in years (SD) (P = .207)*	Gender (F:M) (P = .395)*
Fibroma	D3S1300 (3p)	5	1 (20)	1 (20)	2 (40)				
	D9S1748 (9p)	10	3 (30)	2 (20)	5 (50)				
	TP53 (17p)	7	0 (0)	0 (0)	0 (0)				
Oral lichen planus	D3S1300 (3p)	8	1 (13)	1 (13)	2 (25)				
	D9S1748 (9p)	10	1 (10)	4 (40)	5 (50)				
	TP53 (17p)	10	1 (10)	0 (0)	1 (10)				
Low-grade dysplasia	D3S1300 (3p)	10	2 (20)	5 (50)	7 (70)				
	D9S1748 (9p)	10	5 (50)	1 (10)	6 (60)				
	TP53 (17p)	9	6 (67)	1 (11)	7 (78)				
High-grade dysplasia	D3S1300 (3p)	10	3 (30)	3 (30)	6 (60)				
	D9S1748 (9p)	10	1 (10)	4 (40)	5 (50)				
	TP53 (17p)	10	4 (40)	4 (40)	8 (80)				
Squamous cell carcinoma	D3S1300 (3p)	9	0 (0)	8 (89)	8 (89)				
	D9S1748 (9p)	10	3 (30)	5 (50)	8 (80)				
	TP53 (17p)	10	3 (30)	6 (60)	9 (90)				
						10/10 (100)	9/10 (90)	60.9 (18.5)	6:4

AI, allelic imbalance; LOH, loss of heterozygosity; MSI, microsatellite instability.

* One-way analysis of variance.

Table IIIAllelic imbalance *P* values at any locus

	Fibroma	OLP	LG	HG
Fibroma				
OLP	.315			
LG	.135	.065		
HG	.135	.065	.526	
SCC	.043	.016	.5	.5

OLP, oral lichen planus; LG, low-grade dysplasia; HG, high-grade dysplasia; SCC, squamous cell carcinoma.

Table IVAllelic imbalance *P* values at more than 1 locus

	Fibroma	OLP	LG	HG
Fibroma				
OLP	.395			
LG	.003	.011		
HG	.009	.032	.348	
SCC	<.001	.003	.395	.248

OLP, oral lichen planus; LG, low-grade dysplasia; HG, high-grade dysplasia; SCC, squamous cell carcinoma.

Table VAllelic imbalance *P* values by marker *D3S1300* (3p)

	Fibroma	OLP	LG	HG
Fibroma				
OLP	.392			
LG	.239	.069		
HG	.326	.134	.325	
SCC	.089	.013	.279	.163

OLP, oral lichen planus; LG, low-grade dysplasia; HG, high-grade dysplasia; SCC, squamous cell carcinoma.

Table VIAllelic imbalance *P* values by marker *D9S1748* (9p)

	Fibroma	OLP	LG	HG
Fibroma				
OLP	.344			
LG	.315	.315		
HG	.344	.344	.315	
SCC	.146	.146	.244	.146

OLP, oral lichen planus; LG, low-grade dysplasia; HG, high-grade dysplasia; SCC, squamous cell carcinoma.

Table VIIAllelic imbalance *P* values by marker *TP53* (17p)

	Fibroma	OLP	LG	HG
Fibroma				
OLP	.588			
LG	.003	.005		
HG	.002	.003	.418	
SCC	<.001	<.001	.372	.395

OLP, oral lichen planus; LG, low-grade dysplasia; HG, high-grade dysplasia; SCC, squamous cell carcinoma.