

Fluorescence Visualization Detection of Field Alterations in Tumor Margins of Oral Cancer Patients

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Abstract **Purpose:** Genetically altered cells could become widespread across the epithelium of patients with oral cancer, often in clinically and histologically normal tissue, and contribute to recurrent disease. Molecular approaches have begun to yield information on cancer/risk fields; tissue optics could further extend our understanding of alteration to phenotype as a result of molecular change. **Experimental Design:** We used a simple hand-held device in the operating room to directly visualize subclinical field changes around oral cancers, documenting alteration to fluorescence. A total of 122 oral mucosa biopsies were obtained from 20 surgical specimens with each biopsy being assessed for location, fluorescence visualization (FV) status, histology, and loss of heterozygosity (LOH; 10 markers on three regions: 3p14, 9p21, and 17p13). **Results:** All tumors showed FV loss (FVL). For 19 of the 20 tumors, the loss extended in at least one direction beyond the clinically visible tumor, with the extension varying from 4 to 25 mm. Thirty-two of 36 FVL biopsies showed histologic change (including 7 squamous cell carcinoma/carcinomas *in situ*, 10 severe dysplasias, and 15 mild/moderate dysplasias) compared with 1 of the 66 FV retained (FVR) biopsies. Molecular analysis on margins with low-grade or no dysplasia showed a significant association of LOH in FVL biopsies, with LOH at 3p and/or 9p (previously associated with local tumor recurrence) present in 12 of 19 FVL biopsies compared with 3 of 13 FVR biopsies ($P = 0.04$). **Conclusions:** These data have, for the first time, shown that direct FV can identify subclinical high-risk fields with cancerous and precancerous changes in the operating room setting.

In 1953, Slaughter published a hallmark article in which he emphasized the importance of examining the field surrounding oral cancers for both risk assessment and management of this disease (1). There has been extensive research in this area since then, more recently, using molecular technology. It is becoming increasingly apparent that genetically altered cells could become widespread across the epithelium of patients with oral cancer, into clinically and histologically normal tissue, and that these cells could drive the process of field cancerization (2, 3). In recognition of this, surgeons try to remove oral squamous cell carcinomas (SCC) with a significant

width of surrounding normal-looking oral mucosa, if anatomically allowed. However, the occult disease varies in size and a wealth of evidence suggests that it frequently extends beyond the tumor clearance. This extension may be responsible for the high rate of recurrence of carcinomas at the primary site (~10-30% of cases; refs. 4-9). There is a pressing need to develop new approaches that can be easily used in clinical practice to facilitate the detection of these clinically occult fields.

One such new approach may involve the use of tissue optics. The association of cancer development with the loss of normal tissue autofluorescence has been reported for a number of tissues and organs (10-15). More recently, visual aids using optical methods to detect such loss have been shown to reveal premalignant and malignant lesions that are not detected by unaided eyes (16-18). We have reported the development of a simple hand-held device that facilitated the detection of autofluorescence loss in both visible and occult high-risk oral lesions through direct fluorescence visualization (FV; refs. 17, 18). The interaction of light with tissue has generally been found to highlight changes in the structure and metabolic activity of the areas optically sampled. Specifically, the loss of autofluorescence is believed to reflect a complex mixture of alterations to intrinsic tissue fluorophore distribution, such as the breakdown of the collagen matrix and a decrease in flavin adenine dinucleotide concentration due to tissue remodeling and increased metabolism associated with neoplastic development. Correspondingly, structural changes in tissue morphology associated with neoplastic development

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in both the epithelium and lamina propria (e.g., thickening of the epithelium, hyperchromatism and increased cellular/nuclear pleomorphism, or increased microvasculature), lead to increased absorption and/or scattering of light, which in turn, reduces and modifies the detectable autofluorescence (16, 17, 19, 20).

The objective of this study was to investigate the value of this device in the operating room to delineate field change in autofluorescence around cancers by determining and comparing the histopathologic and molecular changes of margin biopsies that retained normal FV with those margin biopsies that showed a loss of FV. We chose microsatellite analysis for loss of heterozygosity (LOH) at 3p, 9p, and 17p as the molecular analysis, a method used by many international groups to mark clonal spread and possibly predict recurrence (21). A recent study showed that detection of LOH at 3p and/or 9p at prior cancer sites (after tumor removal) was strongly associated with tumor recurrence: samples with such loss had a 26.3-fold increase in the risk of developing second oral malignancy at the site compared with those that retained both of these arms (22). This current study showed a frequent loss of FV of varying distances (up to 25 mm) in clinically normal-looking mucosa surrounding the tumors and a strong

concordance between loss of autofluorescence in tumor margins and the presence of significant histologic change and molecular risk.

Materials and Methods

Patients. Twenty consecutive patients with biopsy-confirmed primary cancer of the oral cavity were accrued to the study as they presented at the British Columbia Cancer Agency between July 2004 and February 2005. Eligibility criteria included the presence of early stage disease (T₀-T₂) scheduled for surgical excision with intent to cure. All the patients were >18 years of age and provided informed consent.

Of the 20 cases in this study, 65% were male, 65% had a smoking history, and 75% were Caucasian. The average age was 58 (36-80 years). Tumor staging was determined from surgical specimens using American Joint Committee on Cancer Staging criteria (23): eight carcinomas *in situ* (CIS, stage 0) and five stage I and seven stage II invasive SCCs (Table 1). Nine of the SCCs were well to moderately well differentiated with the remaining three poorly differentiated. The majority of the tumors were from the tongue (17 of 20, 85%), with one case from the floor of the mouth, and two from the gum.

The FV device. A description of the research FV device and its use is given in Lane et al. (17). Briefly, it consists of a bench-top light source coupled to a hand-held unit for direct visualization. Lesions were

Table 1. Demographics of patients and description of FVL margin biopsies

Patient ID	Tumor stage	Demographics			Histology* and distance to tumor boundary (mm) [†]			
		Age	Gender	Ever smoke	Anterior	Medial	Posterior	Lateral
1	0	51	M	Y		11	11	11
2	0	78	M	Y		5		
3	0	57	M	Y	12			5
4	0	78	M	Y			8	
5	0	37	F	N		5	5	
6	0	52	F	Y	11	3		
7	0	74	M	Y	25		7	
8	0	45	F	N		5		
9	I	74	M	N				
10	I	62	M	Y	3			8
11	I	44	F	N	8		8	
12	I	58	M	Y		11		
13	I	57	M	N	4			
14	II	77	M	Y			22	4
15	II	40	F	N	5		12	
16	II	80	F	Y	4	8	4	4
17	II	66	M	Y	3	10	12	
18	II	44	M	Y	16			
19	II	50	F	N	8		12	
20	II	36	M	Y	4			9

*Histology: Cancer (light blue), high-grade dysplasia (red), low-grade dysplasia (yellow), no dysplasia or cancer (green), not applicable (gray), no extension of FVL beyond clinical tumor.

[†]Distance: values reflect the distance from the boundary of clinically apparent tumor to the FVL boundary (mm).

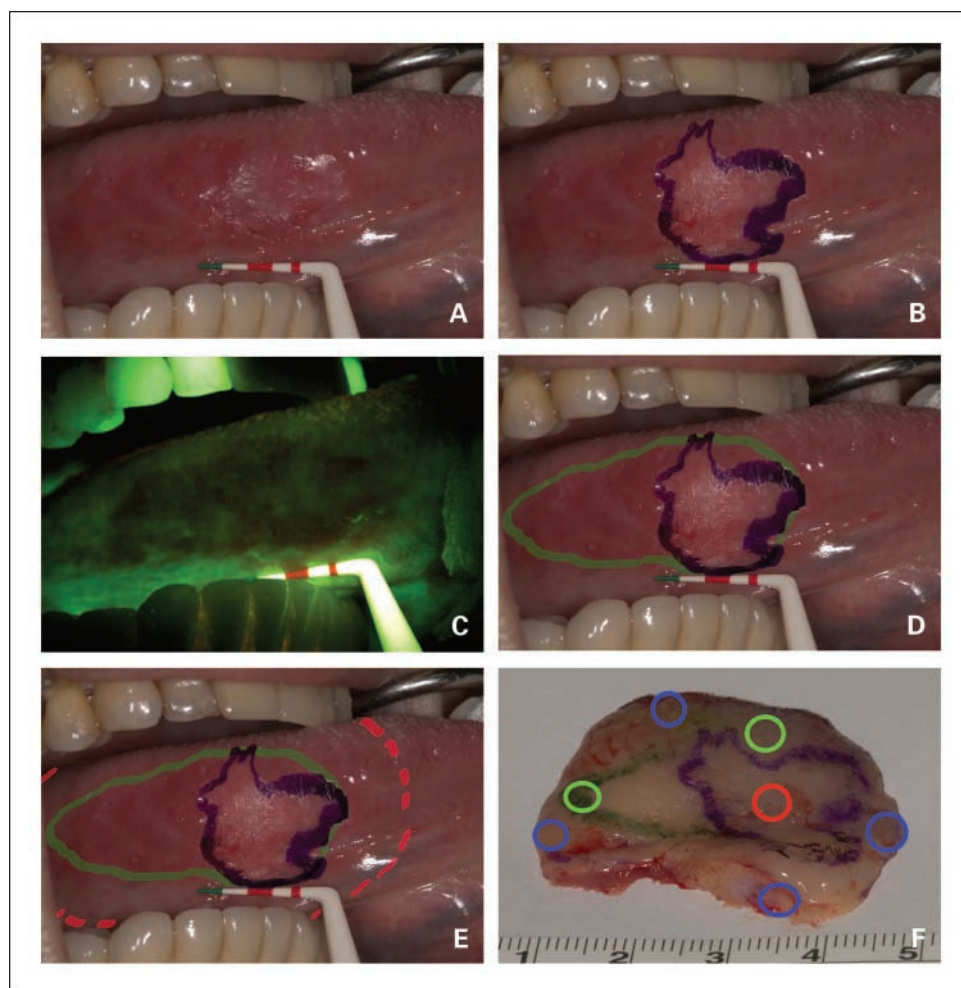


Fig. 1. Stepwise protocol used for assessing surgical field. *A*, in the operating room, initial assessment under white light of an ill-defined SCC at right ventrolateral tongue; *B*, clinically apparent tumor outlined in blue; *C*, assessment of field using FV in the dark; *D*, FVL area outlined in green in the dark; *E*, boundary of surgical specimen (red); *F*, blocking of surgical specimen, showing location of punch biopsy sites from clinically visible tumor (red circle), from tissue showing FVL, placed directly abutting FVL boundary (green circle), and, from tissue showing FVR, placed directly abutting the boundary of surgical specimen (blue circle).

illuminated by this blue/violet light source and then directly visualized through long pass and notch filters, which allow the passage of green and red autofluorescence.

Under direct FV, the normal oral mucosa emits various shades of pale green autofluorescence. Clinical lesions that retained the normal green autofluorescence under FV were defined as FV retained (FVR). Tissue which showed a reduction in the normal pale green and appeared as dark patches were classified as FV loss (FVL; see example in Fig. 1C; ref. 18). This distinction involved a comparison of the lesion site with both adjacent tissue and, as an anatomic control, with tissue on the contralateral side.

Photographs of tissue fluorescence were acquired using illumination from the FV device and a digital single lens reflex camera (Fuji FinePix S2 Pro, Fujifilm, Odawara, Japan) with a long-pass filter (Schott GG475-3, Howard Glass, Worcester, MA). The single lens reflex camera was equipped with a 105 mm f/2.8 macro lens (Nikkor-Micro, Nikon, Tokyo, Japan) and a ring flash (Nikon Macro Speedlight SB-29s, Tokyo, Japan) for white-light images.

Surgical field assessment of FV status. The protocol involved the examination of the surgical site of each patient under both regular operating room illumination and with direct FV, in a stepwise fashion as shown in Fig. 1. All procedures were done while the patient was under general anesthesia and each step was photographed for documentation. The steps included an initial assessment under regular operating room light (Fig. 1A, step 1), demarcation of the boundary of the clinical tumor using a blue marker (Devon skin marker, Ludlow Company, Chicopee, MA) as judged by the surgeon (D.W. Anderson or J.S. Durham; Fig. 1B, step 2), followed by assessment of the site for

altered fluorescence using direct FV (Fig. 1C, step 3). The latter examination was done with the light turned off, using the FV device. Areas showing loss of normal green fluorescence were outlined, demarcating FVL boundaries (Sharpie green marker, Sanford, Oak Brook, IL; Fig. 1D, step 4). Then the light was turned back on, the distances between the clinically visible tumor under white light and FVL boundaries were ascertained using a flexible ruler (Devon skin marker, Ludlow) in four directions: anterior, posterior, medial (to the sagittal plane or dorsum tongue), and lateral (to the sagittal plane or floor of mouth margin). Finally, an electroknife was used to outline the surgical boundary (Fig. 1E, step 5).

Tissue sampling and histologic assessment. After resection, a total of 122 punch biopsies (5 mm) were taken from the tumor and from the tumor margins with at least one margin biopsy from each of the four directions (Fig. 1F, step 6 and Fig. 2). All biopsies were fixed in formalin and submitted for histopathologic evaluation by study pathologists without knowledge of FV status (L. Zhang, R.W. Priddy, and K.W. Borean).

Microsatellite analysis of tumor margins. All FVL biopsies from the tumor margins with a histologic diagnosis of low-grade dysplasia (15 biopsies) or no dysplasia (4 biopsies) were microdissected and analyzed for LOH (Fig. 2, see LOH analysis). As a control, an additional 13 biopsies were analyzed from FVR margins. The protocols for digestion and extraction of samples, LOH analysis, and scoring are described in Zhang et al. (13). All samples were coded so that LOH analysis was done without knowledge of diagnosis or FV status. Microsatellite markers that were used mapped to the following 10 regions: 3p14.2 (*D3S1234*, *D3S1228*, and *D3S1300*), 9p21

(*IFNA*, *D9S171*, *D9S1748*, and *D9S1751*), and 17p11.2 (*CHRN1*) and 17p13.1 (*tp53* and *D17S786*). These were markers used in previous studies to predict cancer risk of oral premalignant lesions (8, 22, 24–28).

Statistical analysis. Differences and associations between groups were examined using either Fisher's exact test for categorical variables or *t* test for continuous variables. All tests were two-sided. *P* < 0.05 was considered to be statistically significant.

Results

A total of 122 oral mucosa biopsies were obtained from the 20 tumors, 20 from the clinical tumor itself and 102 from the tumor margins. Figure 2 shows the study design and summarizes biopsy-specific data obtained for location, FV status, histology, and LOH. For each surgical sample, there were three boundaries: the boundary of the clinically apparent tumor (Fig. 1B), the FVL boundary (Fig. 1D), and the surgical boundary (Fig. 1E). Thirty-six margin biopsies were obtained from FVL tissue and these were placed adjacent to the FVL boundary. The 66 FVR margin biopsies were placed adjacent to the surgical boundary (Fig. 1F).

Novel FVL fields extend beyond the clinical boundary. All tumors showed a loss of fluorescence (FVL), regardless of tumor stage and grade of differentiation. In 19 of 20 tumors, FVL boundaries extended beyond the clinically apparent lesion (Table 1). The extent of this subclinical FVL extension varied considerably, ranging from 4 to 25 mm (mean, 10.3 ± 5.7 mm), with 10 tumors showing a >10-mm FVL extension in one or more directions. It is important to note that FVL extension was never evenly distributed around any given tumor. For example, the tumor in Fig. 1 showed subclinical FVL extension primarily in the posterior direction; in contrast, most of the extension in the tumor in Fig. 3 was in the anterior and lateral directions, with minimal extension in the medial and posterior directions.

To investigate the possibility that the advent of invasion is accompanied by a more aggressive lateral/horizontal subclinical FVL spread, we compared the margin mapping data in the 8 preinvasive high-grade lesions (CIS) with the 12 invasive SCCs. The average width for subclinical FVL extension beyond the clinical boundary was similar for CIS and invasive SCCs (10.4 ± 6.7 versus 10.2 ± 5.6 mm; *P* = 0.79).

FV identifies the majority of histologic risks. As shown in Fig. 2, among the 36 FVL margins, there were 7 (19%) cancers (CIS/SCC), 10 (28%) high-grade dysplasias, 15 (42%) low-grade dysplasias, and 4 (11%) cases with no dysplasia. In contrast, only 1 of the 66 FVR margins was dysplastic. In other words, FVL identified 32 of the 33 cancerous or dysplastic biopsies in the 102 margin biopsies, including all of the cancerous and high-grade dysplasias. There was a significant correlation between the presence of high-grade dysplasia and above with loss of FV (*P* < 0.0001).

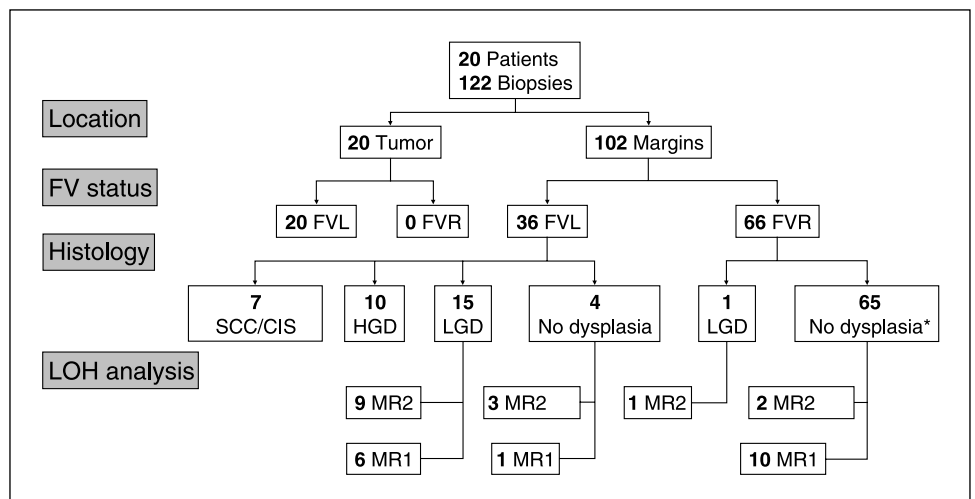
Of the 10 tumors showing >10-mm FVL extension at one or several directions of the tumor margins, 6 tumors showed histologic changes of high-grade dysplasia and above in biopsies taken from FVL regions >10 mm from the clinical tumor boundaries (Table 1: cases 1, 12, 14, 15, 17, and 19).

Molecular risk assessment of low-grade lesions. Because histology is a poor indicator of outcome for margins with little (low-grade) or no histologic change, we used molecular analysis to further define risk for FVL and FVR margins. An example of this combined analysis and its value in assessing FVL margins is shown for case 10 (Fig. 3).

Microsatellite analysis of LOH at 3p, 9p, and 17p was done for 32 biopsies, consisting of all 19 FVL margins showing low-grade dysplasia or no dysplasia, and 13 FVR margin biopsies: the single case with mild dysplasia and 12 randomly chosen cases with no dysplasia (Fig. 2). As shown in Fig. 4A, consistently higher rates of LOH in all categories of comparisons were observed in FVL margins as compared with FVR margins. Such higher rates were significant at 9p (53% versus 8%, *P* = 0.01), for >1 arm lost (37% versus 0%, *P* = 0.03), for LOH at 3p and/or 9p only (63% versus 23%, *P* = 0.04), and for 3p and/or 9p plus 17p (37% versus 0%, *P* = 0.03; Table 2). Strikingly, of the four FVL margins with no dysplasia, two showed LOH at 3p and/or 9p plus 17p, and one showed LOH at 3p (Fig. 2). Of the 13 FVR margins, 3 also showed LOH at 3p and/or 9p, including the single mild dysplasia that was FVR.

As mentioned above, six tumors showed histologic changes of high-grade dysplasia and above in biopsies taken from FVL regions >10 mm from the clinical tumor boundaries. Molecular assessment showed an additional two cases with molecular risk in biopsies taken from FVL regions >10 mm from the clinical tumor boundaries (Table 1, cases 3 and 6).

Fig. 2. Study design showing results of analysis for 122 biopsies. Each biopsy is described with respect to location (tumors, margin), FV status (FVR, FVL); histology [SCC, CIS, high-grade dysplasia (HGD), low-grade dysplasia (LGD); no dysplasia], and LOH analysis [presence of patterns previously associated with recurrence; ref. (22): MR1, no LOH at 3p and 9p; MR2, LOH at 3p and/or 9p]. *, 12 cases were randomly selected from FVR margins without dysplasia for LOH analysis.



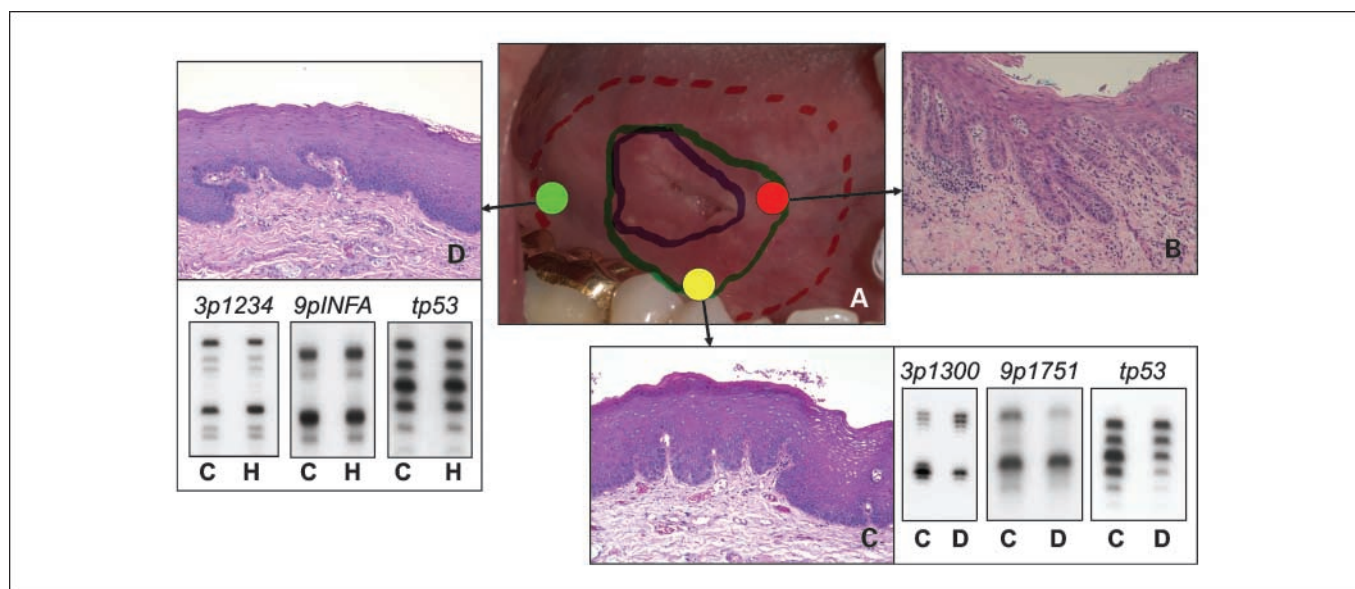


Fig. 3. Presence of high-grade histology or molecular clones in FVL margins outside of clinically apparent tumor. *A*, mapping of surgical field showing three boundaries: clinically apparent tumor (*blue*), FVL boundary (*green*), and boundary of surgical specimen (*red*). *B*, photomicrograph of FVL margin (*red circle*) showing high-grade dysplasia. *C*, photomicrograph and LOH images of FVL margin (*yellow circle*) showing mild dysplasia with LOH at *D3S1300*, *D91751*, and *tp53*. *D*, photomicrograph and LOH images of FVR margin (*green circle*) showing no dysplasia and heterozygosity (no LOH) at *D3S1234*, *D91NFA*, and *tp53*. Magnification, $\times 100$.

Discussion

Molecular technology has begun to shed new light on the definition of “field-at-risk” in patients with oral cancer. In this study of fluorescence field changes, we show that the development of new optical techniques that enable us to visualize spectral alterations associated with oral cancer could add a further dimension to these developing paradigms regarding the concept of cancer/risk field.

Our data indicate strongly that the field of FV alterations (FVL) within or beyond the clinically apparent tumor area is associated with morphologic high-grade and molecular high-risk tissue change. All the 20 tumors in this study displayed FVL. All but 1 of the 36 margin biopsies from the subclinical FVL field had either histologic dysplasia/cancer and/or genetic alterations associated with molecular risk. Seventeen of the 36 cases (47%) had cancer or severe dysplasia and 15 cases (42%) had low-grade dysplasia. Nine of the 15 latter cases showed LOH at 3p and/or 9p, a molecular pattern associated with a 26-fold increase in relative cancer risk for tumor recurrence (29). Only 4 of the 36 (11%) FVL margins were not dysplastic; however, three of the four biopsies showed LOH at 3p and/or 9p when assessed molecularly. In contrast, only 1 of the 66 FVR margins was dysplastic (low-grade) and 3 of the 13 FVR margins analyzed for LOH showed molecular risk (includes the dysplastic case).

These findings add to the growing evidence that supports the use of FV to detect cancers and high-risk lesions (16, 30–32), including occult or nonapparent lesions/areas (18). The closest report existing in the literature to our present study is that of Svistun et al. (16) in which the authors evaluate a similar visual analysis system on excised oral cancer tissue and surrounding tissue *ex vivo*. The best subset of the illumination and detection wavelengths found in their study is identical to the ones used by the FV device in the present study. Although they had a small number of cases (four), their limited results indicated a corre-

spondence between pathology and abnormal fluorescence. A limitation of the study, however, was the use of excised tissue and the identification of areas of altered fluorescence by a surgeon using pictures of this tissue under different conditions.

One of the most difficult and contentious issues with respect to treatment of oral cancers involves the decision on the width of clinically normal tissue that should be removed in addition to the tumor. In an effort to remove occult high-risk field change, surgeons frequently remove an arbitrary 10 mm or more of normal-looking mucosal margin when excising oral cancer, if anatomically possible. Unfortunately, this approach still fails to completely remove the occult high-risk field changes in many patients, resulting in a high-rate of tumor recurrence. Our data showed that such occult change is a frequent event (found in 19 of the 20 tumors), and that the width of this subclinical extension varies considerably (4-25 mm), frequently extending in at least one direction by

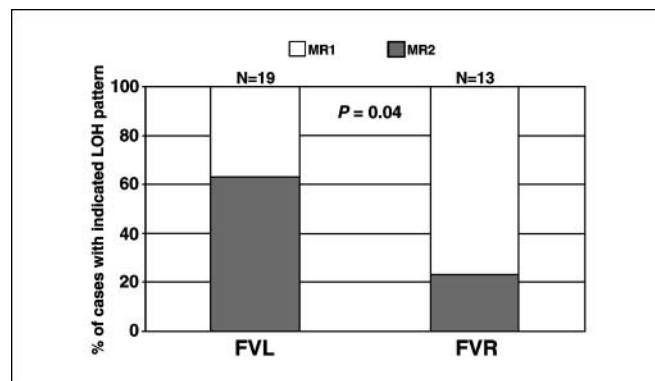


Fig. 4. LOH status of FVL and FVR margin biopsies. Relative frequencies of two molecular patterns previously associated with recurrence (22): MR1, no LOH at 3p and 9p (*open columns*); MR2, LOH at 3p and/or 9p (*solid columns*; see Fig. 2).

Table 2. Frequencies of occurrence of other LOH patterns

	FVL (%)	FVR (%)	P
No. of margin biopsies	19	13	
LOH at individual arms			
at 3p	5 of 19 (26)	2 of 13 (15)	0.67
at 9p	10 of 19 (53)	1 of 13 (8)	0.01
at 17p	7 of 19 (37)	1 of 13 (8)	0.1
Any loss	12 of 19 (63)	4 of 13 (31)	0.15
>1 loss (as ≥ 2 loss)	7 of 19 (37)	0 of 13 (0)	0.03
LOH at 3p and/or 9p	12 of 19 (63)	3 of 13 (23)	0.04
LOH at 3p and/or 9p plus 17p	7 of 19 (37)	0 of 13 (0)	0.03

>10 mm (Table 1). If a 10-mm clearance of clinical tumor was used arbitrarily in this sample set, half of the 20 tumors in this study would have cancer or dysplasia at the surgical margin, with six cases (30%) showing severe dysplasia or CIS. These six tumors would have a high chance of tumor recurrence because of the inadequate removal.

The present study is the first description of an FV-characterized field as ascertained directly in a clinical surgical setting. As such, the data represents a new phenotype that could contribute significantly to our concept of cancer/risk field. More research is required to further define it biologically and clinically. In addition to histology, this report has integrated FV status with molecular changes to assess the cancer/risk field. There was a strong association of LOH with FVL; however, this molecular change was also present in 3 of 12 FVR margins. These data illustrate the complexity of the cancer field and support the need for a multiparameter assessment of such change. Optical devices

and molecular techniques could complement each other. For example, surgical margins of oral cancer have been examined intraoperatively using quantitative methylation-specific PCR and methylation-positive margins have been identified (33). Optical devices could enhance this molecular mapping. In turn, the assessment of FVL boundaries for such molecular change or others (e.g., *p53* mutation with mutation-specific plaque hybridization assay; ref. 5) would improve our understanding of the nature of this new phenotype. It should be noted that the need for multiparameter assessment of the cancer field also includes the development of new approaches to assessing the depth of cancer extension *in vivo*, as the current device assesses mainly lateral cancer spread.

Finally, our data found no difference between CIS and invasive SCC in terms of the FV field expansion. The information is important because the usual recommendation for preinvasive high-grade lesions tend to be more conservative with smaller margins of normal-looking mucosa. The study results suggest that a subgroup of these preinvasive lesions may have extensive lateral fields, some occult, and as such, would require a more aggressive therapy.

In summary, the current study is an important step in the development of a potential integration of optical technology into the management of patients with oral cancer. The device will need to be integrated with information from other sources, both histologic and molecular, and experience with the device will have to be associated with clinical outcome before its clinical value can be established. However, as a proof-of-principle, our data has, for the first time, shown that direct FV can identify subclinical high-risk fields with cancerous and precancerous changes in the operating room setting.

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