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# Histopathological determinants of autofluorescence patterns in oral carcinoma

Running title: histopathology and fluorescence in oral cancer

Keywords: oral carcinoma; autofluorescence; early diagnosis; fluorophores.

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## Introduction

Current gold standard for oral squamous cell carcinoma (OSCC) and potentially malignant disorders (PMDs) diagnosis is histopathologic analysis, usually performed on preoperative incisional biopsies. Several tools have been proposed in order to improve the diagnostic accuracy and to possibly help the identification of areas most suspicious of dysplastic or neoplastic alterations (Holmstrup, Vedtofte, Reibel & Stoltze, 2007). However, the use of such diagnostic aids is associated to a somewhat low sensitivity and/or specificity (Giovannacci, Vescovi, Manfredi, & Meleti, 2016).

Biologic tissues (including oral mucosa) can absorb and re-emit specific light wavelengths, detectable through spectrophotometric devices. Such a phenomenon is known as “autofluorescence” (AF) (Croce, & Bottiroli, 2014).

Several devices evaluating tissues AF have been developed and commercialized in the last two decades (Nazeer, Asish, Venugopal, Anita, & Gupta, 2014; Jayanthi *et al.*, 2009; Lane *et al.*, 2006; Kikuta, 2018). Among these, the Velscope<sup>®</sup> system has been proposed as visual diagnostic aid for PMDs and malignant lesions of the oral mucosa (Morikawa, Kosugi, & Shibahara, 2019).

Clinically healthy oral mucosa, evaluated through the Velscope<sup>®</sup> system appears bright green (normofluorescent). By contrast, specific alterations of the mucosal architecture (including presence of malignant lesions) may display a wide range of AF patterns, varying from hypo fluorescent (dark) to hyperfluorescent (very bright) (Yamamoto *et al.*, 2017; Cicciù *et al.*, 2017). However, the usefulness of Velscope<sup>®</sup> might be somewhat questionable, as the loss of AF is not restricted to neoplastic lesions (Morikawa *et al.*, 2019). Furthermore, contrarily to expectations, some malignant lesions may have a hyperfluorescent pattern. It is therefore evident a lack of knowledge on the specific histopathologic features associated to AF variations.

In the present pilot study, we investigated which are the main histopathological features possibly related to variations of AF patterns in a set of oral SCC and verrucous carcinoma (VC).

## Materials and Methods

The study was approved by the Ethical Committee of the Academic Hospital of Parma (n°46556/17).

Twenty oral lesions (2 VC and 18 SCC) from 15 patients (males:5; females: 10; mean age: 69 - min: 39, max: 90) were included. All lesions were evaluated with regard to AF features, through the Velscope<sup>®</sup> system, before excisional or incisional biopsy. Lesions were classified as normofluorescent, hypofluorescent or hyperfluorescent (Table 1).

Eight histological categories were investigated, in H&E sections, in order to identify which histopathological features are possibly related to the pattern of AF: a) mean width of the entire epithelium (MWE); b) mean width of the keratin layer (MWK); c) mean width of the epithelium without taking into account the overlying keratin; d) overall area of the epithelium (OAE); e) mean depth of inflammatory infiltrate (MDI). For each specimen, severity of inflammatory infiltrate was taken into account (classified as “mild”, “moderate” and “severe”); f) overall area of blood vessels (OAV); g) mean area of blood vessels (MAV) and h) mean diameter of blood vessels (MDV) (Figures 1 and 2). Evaluations were performed through the Nikon NIS-Elements software (3.06 version). All measures were expressed in  $\mu\text{m}$  and taken at 4X magnification except for OAV, MAV and MDV which were taken at 10X magnification.

Both parametric tests (Student's t-test) and non-parametric tests (Mann-Whitney U-test) were used for groups comparisons between the continuous variables (results significant for p-values <0.05). Binary logistic regression was used to test the possible association between fluorescence expression (hyper, hypo) and the main covariates and factors.

## Results

Twelve (60%) lesions were classified as hypofluorescent and 8 (40%) were hyperfluorescent (Table 1).

Severity of inflammatory infiltrate was homogeneously distributed among hypo fluorescent and hyperfluorescent cases ("mild": 3 in hypo and 3 in hyper; "moderate": 3 in hypo and 3 in hyper; "severe": 4 in hypo and 4 in hyper).

Among all the histological features, only MKW showed a strong statistical association with AF ( $p < 0.001$ , for all tests). Particularly, hypofluorescent lesions had a decreased width of the keratin layer (mean MKW:  $41.3 \mu\text{m}$  - SD: 34.4; SE: 9.93) when compared to hyperfluorescent specimens ( $197 \mu\text{m}$  - SD: 69.9; SE: 24.7).

A mild trend toward significance was observed also for MAV ( $p = 0.094$  - Student's test). In fact, mean MAV in hyperfluorescent lesions was  $3755 \mu\text{m}^2$  (SD: 3697, SE: 1307) and it was  $1711 \mu\text{m}^2$  (SD: 1352, SE: 390) in those hypo fluorescent. Even if not statistically significant ( $p$ : 0.328; 0.397; 0.792), also the MDV was smaller in hypo fluorescent lesions ( $38.8 \mu\text{m}$  vs  $48.2 \mu\text{m}$ ).

A tentative classification produced by a binomial logistic regression model including the predictors MWE, MWK, MAV, and OAV has shown a convergence to a solution only for a model with the variable MWK, with a prediction accuracy of 90% regarding the typology of fluorescence (sensitivity = 87.5% and specificity = 91.7%). None of the other variables was statistically associated to AF.

## Discussion

According to the results of the present pilot study, when different epithelial compartments (cellular and keratin layers) are evaluated separately, keratin is, by far, the main portion of the whole oral epithelium that influences AF patterns. Epithelia from malignant lesions with thicker keratin layers are hyperfluorescent whereas lesions with thinner keratin layers are dark hypo fluorescent.

Taking into account that hyperplasia and increase of keratinization are more frequently observed in PMDs such as leukoplakia, in well-differentiated SCC (e.g. presence of keratin pearls) and in VC (e.g. presence of keratin plugs) it is possible to hypothesize that brighter lesions are presumptively at an early stage of the malignant developmental process. On the contrary, oral malignant lesions with thin epithelial and/or keratin layers (or without keratin at all) are supposedly composed of cells far from their original developmental line (e.g. undifferentiated SCC) and probably to a later stage of malignant development (Burian E, *et al.* 2017).

Blood vessels in hyperfluorescent lesions have a mean area greater than those in hypo fluorescent ones. Taking into consideration possible bias due to the low sample size, it can be hypothesized that a higher quantity of relatively small blood vessels (e.g. phase of (neo)angiogenesis) can, altogether with other factors, be associated to the loss of AF.

In conclusion, even with the possible limitations and bias of the present pilot study, it seems rationale to state that AF features of oral malignant lesions are significantly associated to the width of their keratin layer.

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Table 1. Clinical, histopathological and AF features of 20 cases of SCC and VC.

Specimen	Oral site	Clinical presentation	AF pattern*	Histopathological diagnosis
1	cheek	non homogeneous white and red, harden	hypo-fluorescent	VC
2	cheek	non homogeneous red, harden	hypo-fluorescent	in situ OSCC
3	tongue	non homogeneous red, harden	hypo-fluorescent	in situ OSCC
4	tongue	non homogeneous red, ulcerated	hypo-fluorescent	infiltrative OSCC
5	tongue	homogeneous white, harden	hyper-fluorescent	in situ OSCC
6	gingiva	non homogeneous white and red, harden	hypo-fluorescent	in situ OSCC
7	tongue	non homogeneous white and red, harden	hyper-fluorescent	infiltrative OSCC
8	tongue	non homogeneous red, harden, ulcerated	hypo-fluorescent	in situ OSCC
9	hard palate	homogeneous red	hypo-fluorescent	in situ OSCC
10	gingiva	non homogeneous white and red, harden	hypo-fluorescent	infiltrative OSCC
11	tongue	non homogeneous red, ulcerated	hypo-fluorescent	infiltrative OSCC
12	tongue	non homogeneous red, ulcerated	hypo-fluorescent	in situ OSCC
13	tongue	non homogeneous white, ulcerated	hyper-fluorescent	VC
14	tongue	non homogeneous white and red, harden, ulcerated	hyper-fluorescent	infiltrative OSCC
15	tongue	non homogeneous white and red, harden, ulcerated	hypo-fluorescent	in situ OSCC
16	tongue	non homogeneous red, harden, ulcerated	hypo-fluorescent	infiltrative OSCC
17	tongue	non homogeneous white, harden	hyper-fluorescent	in situ OSCC
18	gingiva	non homogeneous white and red, harden, ulcerated	hyper-fluorescent	microinvasive OSCC
19	soft palate	non homogeneous white and red, harden, ulcerated	hyper-fluorescent	in situ OSCC
20	hard palate	non homogeneous white and red, harden, ulcerated	hyper-fluorescent	in situ OSCC

Legend:

\* AF pattern is specifically referred to the site of where the biopsy was taken VC: Verrucous Carcinoma; OSCC: Oral Squamocellular Carcinoma

## Figures legend

Figure 1. Examples of evaluation of width of epithelium, keratin and area of the epithelium: a) maximum and minimum length of the epithelium; b) maximum and minimum length of the keratin layer; c) overall area of the epithelium in 3 consecutive fields (H&E staining, 4X magnification).

Figure 2. Examples of evaluation of depth of inflammatory infiltration and area of blood vesels: a) maximum and minimum depth of inflammatory infiltration (H&E staining, 4X magnification); b) overall area of blood vessel in one field of observation (H&E staining, 10X magnification).