Review article

Detection of field alterations using useful tools for oral squamous cell carcinoma

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Filed cancerization;
Field alternation;
Dysplasia;
Oral squamous carcinoma

Summary It is known that a region of epithelial dysplasia cannot easily be distinguished macroscopically from normal looking area surrounding early oral squamous cell carcinoma (OSCC). In 1953, Slaughter emphasized field cancerization and the importance of examining the fields surrounding OSCC. Since 1997, we have used vital staining with iodine for detecting surgical margin and investigated the usefulness of this method. From a pathological point of view, various types of dysplasia in iodine unstained area (IU) surrounding OSCC was found. In oral mucosa, iodine—glycogen reaction does not occur in dysplastic mucosa due to the lack of glycogen granules in the cytoplasm of those cells. This area has high positive PCNA and p53 cells with malignant potentiality. More recently, since 2010, we have used fluorescence visualization (FV) with vital staining with iodine. This device can visualize epithelial dysplasia surrounding OSCC as fluorescence visualization loss (FVL). FVL has high positive Ki-67 and p53 cells with malignant potentiality. We suggest that FV has delineated various types of dysplasia and the delineation of surgical margin is the same or better than vital staining with iodine. Moreover, compared to vital staining with iodine, it is simple to use with no invasion.

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1. Introduction

Oral squamous cell carcinoma (OSCC) is a major health problem worldwide, accounting for 274,000 new cases and 145,000 deaths annually [1]. In Japan, it is estimated that there will be 6900 new cases of OSCC in 2005 [2]. Furthermore, there is definitely an increasing number of OSCC as a result of Japan’s aging society [3]. The conventional treatment of early OSCC is surgery, however, early OSCC patients sometimes recurred after radical resection. When surgeons try to remove early OSCC, a region of epithelial dysplasia cannot easily be distinguished macroscopically from normal looking area surrounding OSCC. Leaving this epithelial dysplasia unresected can often result in local recurrence or second primary tumors (SPTs). This may result in the recurrence of carcinomas at the primary site. From a clinical point of view, it may lead to SPTs to establish not only the histology but also the molecular change of the surgical margin. It is likely that SPTs is a result of accumulated genetic changes present in the histologically normal epithelium of the surgical margin [4]. Thus, in contrast to advanced OSCC, some patients with early OSCC who were considered curable eventually suffered from locoregional failure after radical resection. The present article reviews our current understanding on some methods for detecting a safety margin, including field alteration surrounding early OSCC.

2. Field alterations surrounding OSCC (Slaughter’s concept)

Slaughter reported about his concept of field cancerization, when studying OSCC in 1953 [5]. He emphasized the importance of examining the field surrounding OSCC for both risk assessment and management of this disease. By performing extensive histological examinations, he proposed a concept describing issues covered by the term field cancerization as follows (Fig. 1):

a. OSCC develops in multifocal areas of precancerous change;

b. Abnormal tissue surrounds the tumor;

c. Oral cancer often consists of multiple independent lesions that sometimes coalesce;
d. The persistence of abnormal tissue after surgery may explain SPTs and local recurrences.

It is well known that an accumulation of genetic alterations forms the basis for the progression from a normal cell to a cancer cell, referred to as the process of multistep carcinogenesis (Fig. 2). Slaughter suggested that OSCC develops in the following order; from oral premalignant lesions, which is hyperplasia; to mild dysplasia; then to moderate dysplasia; and to severe dysplasia; then into carcinoma in situ (CIS); and finally to invasive SCC. WHO advocated that mild and moderate dysplasia as low grade dysplasia and severe dysplasia and carcinoma in situ as high grade dysplasia [6]. Braakhuis et al. reported that the presence of allelic loss at 3p and 9p is associated with an increased cancer risk. Additional losses at 17p increased cancer risk dramatically [7]. The process of field cancerization can be defined in these allelic losses, and its position in the process of multistep carcinogenesis can be delineated. Furthermore, some reports indicated that the presence of a field change with genetically altered cells is a distinct biological stage in malignant potential with important clinical implications [4,7–11]. Thus, we suggest that various types of dysplasia surrounding OSCC have to be removed completely. However, these mechanisms remain unclear and it is important to establish the method of detecting safety margin in early OSCC.

Figure 1 The concept of field cancerization [5]. The epithelial dysplasia often observed around SCC is considered to cause local recurrence or a second primary cancer. It is not clear to detect outline of this abnormal area by the naked eye (arrow).
3. Vital staining with iodine for the detecting field alternations

As described in Slaughter’s concept, a region of epithelial dysplasia surrounding early OSCC have to be delineated and removed. Since 1997, we have performed not only conventional resection but also iodine staining method for patients with early OSCC. Iodine staining of mucosal lesions was first reported by Schiller who used it to identify cervical cancer of the uterus [12]. Thereafter, this method has been employed for the upper gastrointestinal tract [13–15]. Epstein et al. reported that possible patients of oral premalignant lesions and early oral cancers used vital staining with iodine, and the result of sensitivity was 92.5% and specificity 63.2% [16,17]. Kurita et al. observed that vital staining with iodine is a useful method for identifying malignant lesions and lesions with the possibility of malignancy in Japan [17].

3.1. Comparison between resection of no vital staining and of vital staining with iodine

Since 1982, we have established that a clear margin is defined as the distance from the invasive tumor front that is 10 mm or more from the resected margin, as conventional resection in our clinic (Fig. 3). After that, since 1997, we have used vital staining with 3% iodine solution to decide the surgical margin. Currently, to delineate safety margin in early OSCC, our clinic uses the combination of fluorescence visualization and vital staining with iodine to detect epithelial dysplasia.

With vital staining with iodine, we resected tumors to detect surgical margin of 5 mm distance from iodine unstained area (IU) (Fig. 4). The purpose of our study was to determine the usefulness of vital staining with iodine in deciding the surgical margin. One hundred and two patients diagnosed with T1 and early T2 OSCC between 1987 and 2006 were selected at the Department of Oral and Maxillofacial Surgery, Tokyo Dental College. We have already gotten an official approval from the ethical committee of Tokyo Dental College. We classified the patients into two categories as follows: Group 1, where no iodine staining was carried out, all treated between January 1987 and December 1996 (41 cases); and Group 2, where iodine-staining was carried out, all treated between January 1997 and December 2007 (61 cases). We compared clinical and pathological histories between these two groups. First, in positive rates of second primary cancers, the results revealed that patients in Group 1 (17.1%) were significantly higher than those in Group 2 (4.9%) (Fig. 5A). Next, in positive rates of pathological findings of surgical margin, the epithelial dysplasia-positive rate in Group 1 (26.8%) was higher than that in Group 2 (8.2%) (Fig. 5B). Finally, there were surgical margin-positive in 2 cases in Group 1 (4.9%). We suggest that the iodine staining method is a useful tool in deciding surgical margin in early OSCC.

![Figure 2](image-url)  
**Figure 2** Multistep carcinogenesis of oral malignancy (Slaughter’s concept [5]).

![Figure 3](image-url)  
**Figure 3** History of the detection of surgical margin for early oral cancer in our clinic.
3.2. Pathological analysis of IU surrounding OSCC

A histochemical and ultrastructural study on the localization of glycogen in normal and hyperkeratotic oral epithelium in humans has demonstrated that glycogen content is inversely related to the degree of keratosis, suggesting a role for glycogen in keratinization [18]. Basically, normal tissue would stain brown, while proliferating epithelium would be unstained or would stain poorly [19]. However, there are few reports on the investigation of IU and malignant potentiality. To clarify the correlation between IU and epithelial dysplasia, we examined the existence of glycogen and changes in Proliferating Cell Nuclear Antigen (PCNA) in tumor suppressor gene expression (p53) in the dysplastic epithelium using immunohistochemical and ultrastructural methods. Subjects are thirty cases of T1 and early T2 OSCC. Based on the WHO classification (2005), we classified the epithelial dysplasia histopathologically into thirty specimens with normal mucosa, twenty specimens with mild, twenty three specimens with moderate and twenty three specimens with severe epithelial dysplasia from thirty cases [6]. There were no significant differences between the normal epithelium (43.0%) and the mild dysplasia (45.5%) in terms of the ratios of PAS positive cells and their distribution (Fig. 6 and Table 1). However, PAS staining area of the moderate and severe dysplasia (13.9%) decreased more significantly than those of the normal epithelium (p < 0.01). Furthermore, the distribution and the ratio of PCNA- and p53-positive cells in the moderate (31.1%, 36.0%) and severe dysplasia groups (41.5%, 41.8%) were significantly higher than in normal epithelium and mild dysplasia (p < 0.01) (Fig. 7 and Table 1). In electron microscopic observations, glycogen granules were seen in the cells of normal epithelium but were not seen in the cells of IU (Fig. 8). In conclusion, IU surrounding OSCC has various types of dysplasia. In oral mucosa, iodine–glycogen reaction does not occur in dysplastic mucosa due to the lack of glycogen granules in the cytoplasm of those cells. This area has high positive PCNA and p53 cells with malignant potentiality. Therefore, from the standpoint of molecular biology, we suggest iodine-staining method as strongly useful tool in defining the region of epithelial dysplasia. On the other hand, we are always unable to see a clear margin with IU. It is known that keratinized squamous epithelia, such as the gingiva and the hard palate, were less reactive to iodine. Iodine–glycogen reaction does not occur in epithelial cells with chronic inflammatory change. Furthermore, we sometimes suffer from drug induced reaction such as iodine hypersensitivity. For this reason, we must look for a more convenient and useful tool for the detection of safety margin.

4. Optically guided surgical approach and the outcome of early OSCC

More recently, Pierre et al. have introduced an approach using fluorescence visualization (FV) technology, fluorescence
Figure 6  PAS staining for iodine unstained area (10×). (A) Normal epithelium with H–E staining, (B) normal epithelium with PAS staining, (C) iodine unstained area with H–E staining and (D) iodine unstained area with PAS staining.

imaging device, and VELscope (LED Dental Inc., White Rock, British Colombia, Canada [19]) (Fig. 9). This simple handheld device uses a blue light (400–460 nm) to illuminate a collagen matrix or a flavin adenine dinucleotide (FAD). A selective filter in the eyepiece allows the viewer to directly visualize the pale green autofluorescence that is given off by normal tissue (fluorescence visualization retention, FVR). On the other hand, abnormal tissue shows decreased autofluorescence and appears as a dark brown in comparison with green surrounding normal tissue. This dark brown is the so called fluorescence visualization loss (FVL). FVL is caused by absorbing a specific blue light due to the breakdown of the collagen matrix and decrease in FAD. The oxidized form of FAD is important fluorophores that are good indicators of cellular metabolism. It has been shown that fluorescence intensity due to FAD decreases with dysplastic progression. We have suggested this device as a suitable adjunct for margin delineation since 2010. Therefore we investigated to evaluate the detection of surgical margin comparison between FV and vital staining with iodine, and the accuracy of autofluorescence examination in its ability to delineate high-risk oral mucosal lesions.

4.1. A clinical study about the comparison between FVL and IU

To clarify the usefulness of FV compared to vital staining with iodine, we investigated surgical margin of early OSCC in comparison between FVL and IU. Thirty one cases of T1 and early T2 OSCC patients were examined in this study. At the time of surgery, the operating surgeon outlined the boundary with vital staining with iodine and fluorescence visualization. With the operating room lights off, we examined the lesion and outlined the FVL, measured its size, and recorded data on the surgical tracking sheet. Moreover, we used vital staining with iodine and considered in comparison between FVL and IU, and we resected according to the wider boundary of the outline. As a result, the entire area by FVL

<table>
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<th>Table 1</th>
<th>Positive ratios of PAS, PCNA, and p53 staining with various dysplasia.</th>
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<tr>
<td>Site</td>
<td>n</td>
</tr>
<tr>
<td>Control (normal epithelium)</td>
<td>30</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>20</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>23</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>23</td>
</tr>
</tbody>
</table>

* * p < 0.01 (vs control).
showed various types of epithelial dysplasia (Fig. 10). There were no normal epithelium cells in any of the FVL regions. Furthermore, the ratio of various types of dysplasia is almost equal between FVL (mild 28.6%, moderate 61.9%, severe 9.5%) and IU (mild 35.3%, moderate 58.8%, severe 5.9%). However, one case of carcinoma of tongue (T1N0M0) showed local recurrence after surgery guided FV and underwent more surgery. In delineating ratio of FVL and IU into thirty one early OSCC cases, twenty seven cases of FVL (87.1%) were same as or a little higher than IU (71.0%) (Table 2). We considered that determining the surgical margin based on results of FV would not lead to over surgery, and could help prevent SPTs.

Figure 7  Immunohistochemical staining for iodine unstained area. (A) Normal epithelium with PCNA staining, (B) normal epithelium with p53 staining, (C) iodine unstained area with PCNA staining and (D) iodine unstained area with p53 staining.

Figure 8  Electron microscopic observations. (A) Normal epithelium cell, (B) iodine unstained cell, G: glycogen granules; D: desmosomes; T: tonofilaments; N: Nucleus; bar = 1 μm. Glycogen granules are not visible in moderate dysplasia compared with normal epithelium.
4.2. The expression of ki-67 and p53 in the area of FVL

To elucidate malignant potentiality in FV area, some cases of early OSCC were examined in this study. These materials were obtained from the department of Oral and Maxillofacial Surgery at Tokyo Dental College. Surgical margin were determined at about 5 mm outside the area of FVL. A superficial incision was made with a surgical blade along the boundary line of FVL to mark the clinical borderline within the mucosa. We examined expression of Ki-67 and p53 in the area of FVL by means of immunohistochemical methods from these samples. Although these samples are not enough, positive cells of Ki-67 and p53 seemed to strongly express within the area of FVL with epithelial dysplasia surrounding OSCC (Figs. 11 and 12). On the contrary, Ki-67 and p53 were hardly seen in epithelial tissue out of margin. Therefore, we suggest that FVL has malignant potentiality and FV guided surgical margin might not only be adequate but also to be able to help prevent SPTs genetically.

5. Discussion

Various types of dysplasia surrounding OSCC looks like a normal oral mucosa. Taking margins that are too large cause severe cosmetic and functional morbidity and margins that

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Cases (n)</th>
<th>FVL</th>
<th>IU</th>
</tr>
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<tbody>
<tr>
<td>Low grade dysplasia</td>
<td>28</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>High grade dysplasia</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>31</td>
<td>27</td>
<td>22</td>
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Delineating ratio (%)

<table>
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<tr>
<th>Diagnosis</th>
<th>Cases (n)</th>
<th>FVL</th>
<th>IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade dysplasia</td>
<td>28</td>
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<td>20</td>
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</tr>
<tr>
<td>Dysplasia</td>
<td>31</td>
<td>27</td>
<td>22</td>
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FVL: fluorescence visualization; IU: iodine unstained area.
are too small may leave cancerous tissue behind, as evidenced by frequent positive surgical margins and high locoregional recurrence. The NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) allows many systemic therapy options for patients with oral cavity cancer in 2012 [20]. NCCN guideline suggested that a margin of at least 5 mm of histologically normal epithelium in the surgical specimen is traditionally regarded as the standard in the treatment of OSCC. Conversely, some reported that such an approach still fails to completely remove the field alterations surrounding OSCC [21–26]. Kurita et al. have also proposed that a 5 mm border of normal tissue peripheral to the iodine positive area

**Figure 11** The expression of ki-67 in the area of FVL. C: cutting off margin of FVL; FVL: fluorescence visualization loss; FVR: fluorescence visualization retention.

**Figure 12** The expression of p53 in the area of FVL. C: cutting off margin of FVL; FVL: fluorescence visualization loss; FVR: fluorescence visualization retention.
would be adequate for the complete removal of the dysplastic epithelium [17]. We have been successful in improving the outcome of the incident of SPTs with the use of iodine-staining method in deciding the margin. PCNA and p53 are known as markers for the malignant potential of the oral mucosa; and PCNA is valuable as a marker to judge biological malignancy and proliferation [27–30]. The p53 mutant gene plays a significant role in malignant transformation [27,31–37]. The positive ratio of PCNA and p53 in moderate and severe dysplasia were higher. We surmise that a mutant p53 appears in the epithelial dysplasia such as an IU area. We are able to obtain the evidence of vital staining with iodine as useful tool for identifying malignant potential tissue surrounding early OSCC. On the other hand, vital staining with iodine produces a brown stain as a result of the reaction of iodine with glycogen. Iodine solution was usually prepared using 1–5% or Lugol’s solution [16,17,37,38]. We have always used 3% iodine solution. However, this method does not allow us to see a clear margin. Epstein et al. and Silverman pointed out that keratinized squamous epithelia or inflammation tissue were less reactive to iodine and useless [16,18]. These problems may limit the use of iodine solution. Thus, simple device, FV may make up for the shortcomings of iodine staining method. It is likely that this device has delineated various types of dysplasia and delineation of surgical margin is the same or more than vital staining with iodine. It is simple to use and no invasion is seen, compared to vital staining with iodine. This device was reported to achieve a sensitivity of 98% and specificity of 100% when discriminating normal mucosa from severe dysplasia/carcinoma in situ or invasive carcinoma [39]. Pierre et al. envisaged this device as a suitable adjunct for oral cancer screening, biopsy guidance, and margin delineation [19,39]. In the resection specimen with cancer or precancerous lesions, microsatellite analysis of LOH at 3p, 9p and 17p was done, and the area of FVL showed higher rates of LOH in all categories significantly [39]. These results are likely to be similar to Slaughter’s concept. Meanwhile some controversy exists. Awan et al. reported that this device demonstrated a relatively high sensitivity (84%) and low specificity (15%) in discriminating high risk dysplasia from benign lesions and was not enough for detection of early diagnosis [40]. Both vital staining with iodine and FV has completely different mechanisms. When FVL is detected only by VELSscope, this lesion would need a careful consideration because of the absence of clinical evidence. Today, as far as this device is concerned, the detection of surgical margin using both FV and vital staining with iodine would be better. In the near future, we will need to investigate about the usefulness of FV with more data.

Conflict or interest

The authors declare no conflict of interest.

Acknowledgements

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