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Title: Intraoperative tissue staining of invaded oral carcinoma

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Abstract: The purpose of this study was to assess the ability of intraoperative tissue staining with consecutive application of 0.4% indigo carmine and 0.5% Congo red to demonstrate the extent and border of oral carcinoma invasion. Seventeen patients were included in the study. Once the oral tumor was resected, a vertical section of surgical specimen was taken from the central part of the tumor. The extent and border of the invaded carcinoma were assessed on digital microscopic examination with tissue staining. The results of assessments were compared with corresponding results of conventional histopathological analysis with HE staining, which is considered the gold standard. Tissue staining produced a brown-black stain on normal muscle, connective, and salivary tissues but not tumor and epithelial tissues. It clearly demonstrated the extent and border of tumor invasion in 13 of 17 patients (76.5%); however, detection of remnant vital tumor cells in scar tissue after neoadjuvant chemotherapy, and distinction between the tumor and adipose tissue scattered in the muscle tissue was difficult. The results of this study showed that intraoperative tissue staining was a possible method in demonstrating the extent and border of carcinoma deeply invaded in the soft tissue and selecting the site for additional frozen section analysis, although the method needed some refinement.
Key words: intraoperative, surgery, tissue staining, invasion, surgical margin

Introduction

One of the most important but difficult aspects of cancer surgery is ensuring complete removal of the tumor at the primary site. However, no reliable intraoperative procedure, other than frozen-section analysis [1-4], has yet been developed to assess complete tumor resection especially in deep surgical margins. Intraoperative macroscopic or digital microscopic examination of resected specimens [5] has been a useful method to assess the extent and border of the invaded carcinoma; however, the procedure had a major problem. The distinction between carcinoma and the surrounding tissue was primarily based on a difference in their colors. An additional method allowing clear distinction of tumor tissues from surrounding healthy tissues is anticipated.

Recently, we developed intraoperative tissue staining to delineate the extent and border of the invaded carcinoma. The consecutive application of 0.4% indigo carmine and 0.5% Congo red highlighted a difference between the invaded carcinoma and the surrounding healthy tissues. The aim of this report was to discuss the ability of intraoperative tissue staining of surgical specimens to delineate the extent and border of invaded oral carcinoma.

Materials and methods

A prospective study of 17 patients who underwent surgery for oral carcinoma was undertaken. Patients with lesions on the gingiva and hard palate were excluded because intraoperative sectioning of the resected hard tissue (bone) was difficult. Informed consent was obtained from each patient. The patients were six women and eleven men, with a mean age of 66.7 years (range 49 – 84). Clinical and histopathological characteristics of the tumors are shown in Table 1. Two patients with recurrent tumor after interstitial radiotherapy were included. Six additional patients underwent platin-based multi-agent neoadjuvant chemotherapy four weeks prior to the planned surgery. Informed consent was obtained from all patients and/or gradients.

A flow chart of the series of examinations is presented in Figure 1. All patients underwent surgical resection of their oral tumor under general anesthesia. Once the tumor was resected, a 5 mm-thick vertical section of the surgical specimen was taken from the central part of the tumor. The section was then pinned to a board and prepared for subsequent examinations.

Firstly, the section was macroscopically and microscopically examined and recorded using a digital microscope system (VHX-1000, KEYENCE Co., Osaka, Japan) without tissue staining. The VHX-1000 has a 1/2-inch, 2.1-million-pixel CCD image sensor and a high-definition, 15-inch HD TFT color LCD monitor (1,600 x 1,200 pixels). In this study, a standard zoom lens with a magnifying power of 25 to 175 (VH-Z25, KEYENCE, Osaka, Japan) was used. Thereafter, tissue staining of the surgical specimen was performed as shown in Table 2. The stained reaction was examined and recorded using the digital microscope system in the same way. The sliced section was then processed for conventional histopathological evaluation of the surgical specimens. The section, pinned to the board, was subjected to formalin fixation. After fixation, the section was embedded in paraffin and a horizontal slice of the section was prepared for conventional microscopic examination with hematoxylin and eosin (HE) staining. The prepared specimen was examined using a light microscope with magnification powers of 40 and 100.

The results of assessments with or without vital staining were compared with corresponding results of conventional histopathological analysis with HE staining, which is considered the gold standard.

Results

Surgical specimens with and without tissue staining, and an HE-stained section are shown in Figure 2 and 3. The tissue staining produced a brown-black stain on normal muscle,

connective, and salivary tissues but not tumor and epithelial tissues. Adipose tissue was usually stained with the solutions. The detection of intraepithelial spread of the tumor was impossible. There was no difference in the stain reaction between the dysplastic/cancerous and normal epithelium. In addition, it must be noted that the tissue staining had no negative influence on subsequent histopathological examination with HE staining.

The results of assessment on digital microscopic examination with and without tissue staining concerning the ability to delineate the invaded tumor are summarized in Table 3. In the examination without tissue staining, the extent of carcinoma invasion was clearly delineated in 10 out of 17 patients (58.8%); however, a distinction between adipose and carcinoma tissue was difficult in four cases, and a distinction between salivary and carcinoma tissue was also difficult in two cases. In three cases, fibrous and scar tissues were misjudged as carcinoma. On the other hand, tissue staining clearly demonstrated the extent and border of tumor invasion in 13 of 17 patients (76.5%); however, remnant vital tumor cells in scar tissue after neoadjuvant chemotherapy could not be detected with tissue staining examination in two cases (Fig. 3). In another two cases, the distinction between adipose tissue scattered in the muscle tissue and the invaded carcinoma was difficult (Fig 4).

Discussion

The purpose of this study was to assess the ability of intraoperative tissue staining of surgical specimens to demonstrate the extent and border of deeply invaded oral carcinoma.

In this study, tissue staining with 0.4% indigo carmine and 0.5% Congo red was employed to demonstrate the extent or border of tumor invasion. Indigo carmine is a conventional contrast dye used for vivid accentuation of the intestinal mucosa. [6,7] If indigo carmine is applied to the surface of the epithelium, the blue dye is not absorbed and collects in the sulci and grooves of the mucosa, highlighting the topography of the stained mucosa. Firstly, we used this solution to produce contrast staining between tumor tissues and the surroundings. It was speculated that the blue dye would pool in the surrounding connective and muscle tissues, thereby delineating the epithelial malignant tissues; however, indigo carmine pooled in the pits of both the tumor and surrounding tissues, failing to distinguish tumor tissue from the surrounding tissue.

Congo red is a reactive pH-dependent coloring agent. It identifies acid-secreting gastric cells and causes the color in these mucosal regions to change from red to dark blue.[6, 7] Congo red is also used to improve the diagnosis of early stomach carcinoma. In consecutive application with methylene blue, stomach areas altered by the tumor are bleached out, thus making them distinguishable from their surroundings.[8] In this study, Congo red was applied after indigo carmine. This consecutive staining enabled demarcation of the extent of tumor invasion; that is, carcinoma tissue was bleached out, while the surrounding connective and muscle tissues were stained brown-black. Thereafter, intraoperative tissue staining of surgical specimens was assessed using consecutive application of these two agents.

In this study, consecutive application of 0.4% indigo carmine and 0.5% Congo red enabled demarcation of the extent of the invaded carcinoma. The tissue staining produced a brown-black stain on normal muscle, fibrous/scar, salivary, and most adipose tissue, but not the invaded tumor, showing the extent of tumor invasion. The results of this study showed that the ability to delineate the invaded tumor in digital microscopic examination was higher with than without tissue staining. The addition of tissue staining enabled clear distinctions between the tumor tissue and either the fibrous/scar, salivary, or adipose tissue, which were difficult to assess in the examination without tissue staining.

However, this tissue staining method had some limitations. As shown in the results of this study, digital microscopic examination with tissue staining could not detect remnant vital tumor cells in fibrous/scar tissue after neoadjuvant chemotherapy. In this study, almost all of

the tumor invaded as a connected cluster of the tumor cells and was clearly delineated with tissue staining. Scattered tumor cells were overlooked in patients who had undergone preoperative treatment. In this study eight patients had undergone preoperative treatments. In six of those, the remnant tumor existed as one entity and was successfully defined while, in the other two cases, vital tumor cells remained in the scar tissue produced by cytotoxic chemotherapy were difficult to be detected. These results suggested that the presence of previous treatment that would produce tumor necrosis and subsequent scar formation might have a negative influence on digital microscopic examination with tissue staining.

It was also difficult to distinguish carcinoma from adipose tissue scattered in the muscle tissue. Most of the adipose tissues were stained brown-black with the consecutive application of 0.4% indigo carmine and 0.5% Congo red. However, adipose tissues scattered in the muscle tissue were not always stained and were misdiagnosed as invaded carcinoma in two of the 17 cases. The results suggested that staining could not make a clear difference between the tumor and adipose tissue, especially if adipose tissues were scattered in the surrounding tissue. Additional methods that can clarify the difference between carcinoma and adipose tissue will be necessary.

In conclusion, the results of this study showed that intraoperative tissue staining of the surgical specimen was a useful method to demonstrate the extent and border of carcinoma deeply invaded in the soft tissue, although the method had some limitations. Suggested limitations were: inability to detect microscopic nests of infiltrating carcinoma, apparent unreliability for resections following chemo/radiation therapy, and ineffectiveness in assessing mucosal margins. And, an additional method that can clarify the difference between the invaded tumor and scattered adipose tissue is necessary.

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Figure Legends

Figure 1. Flow chart of a series of examination. Tissue staining and subsequent histopathological assessment was carried out using a vertical section of the surgical specimen

Figure 2. Surgical specimen resected from patients with tongue carcinoma (case no. 1). (a) Before tissue staining; (b) After tissue staining; (c) Hematoxylin and eosin-stained section. Invaded carcinoma (arrow heads) was clearly defined with tissue staining. Distinguishing fibrous and adipose tissue (circled) from invaded carcinoma was difficult without tissue staining, while it was easy with tissue staining. The fibrous and adipose tissue being stained.

Figure 3. Surgical specimen resected from a tongue carcinoma patient who underwent preoperative chemotherapy (case no. 9). (a) Before tissue staining; (b) After tissue stainig; (c) Hematoxylin and eosin-stained section. Invaded carcinoma (arrow heads) was clearly defined with tissue staining. Distinguishing fibrous/scar tissue (arrow heads), salivary tissue (small circled), and adipose tissue (big circled) from invaded carcinoma was difficult without tissue staining. The fibrous/scar, salivary, and adipose tissue were stained; however, the detection of remnant carcinoma cells scattered in fibrous/scar tissue was difficult.

Figure 4. Surgical specimen resected from a tongue carcinoma patient who underwent preoperative chemotherapy (case no. 16). (a) After tissue staining; (b) Hematoxylin and eosin-stained section. Distinguishing remnant carcinoma cells (arrow heads) and adipose tissues scattered in the muscle tissue (circled) was difficult.

Table 1 Clinical and histopathological characteristics of the tumor

Primary site	
Tongue	13
Oral floor	3
Cheek mucosa	1
Tumor stage (TNM-classification of the UICC)	
T1	7
T2	3
T4	5
rT2	2
Hisopathological diagnosis	
Well differentiated SCC	11
Moderately differentiated SCC	3
Poorly differentiated SCC	1
Early invasive SCC	2
Previous and/or preoperative treatment of the tumor	
None	9
Neoadjuvant chemotherapy	6
Interstitial radiotherapy	2

SCC: squamous cell carcinoma

Table 2 Sequence of dye application

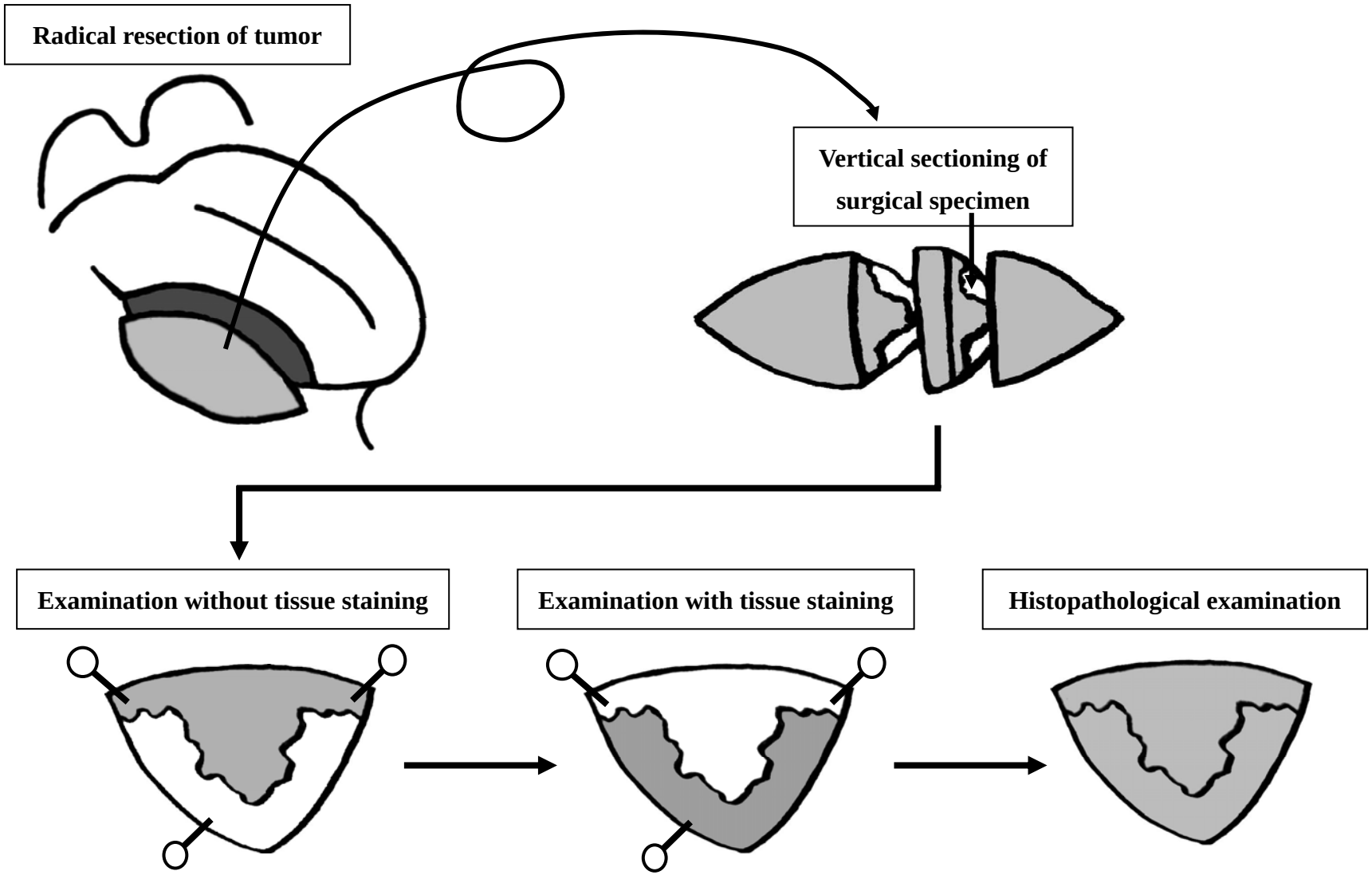
1. Direct examination
2. Gently wash with saline solution and dry with a cotton bud
3. Apply 0.4% indigo carmine with a cotton bud (10 to 20 seconds)
4. Apply 0.5% Congo red with a cotton bud (10 to 20 seconds)
5. Wait for 1 to 2 minutes
6. Interpret stain reaction

Table 3 Ability to delineate invaded carcinoma

No.	Site	Preoperative treatment	Assessment without tissue staining	Assessment with tissue staining
1	Tongue	None	Mistake fibrous/adipose tissue as tumor	Clearly delineated
2	Oral floor	None	Clearly delineated	Clearly delineated
3	Oral floor	Chemotherapy	Clearly delineated	Clearly delineated
4	Tongue	None	Clearly delineated	Clearly delineated
5	Cheek mucosa	None	Clearly delineated	Clearly delineated
6	Tongue	None	Clearly delineated	Clearly delineated
7	Tongue	None	Clearly delineated	Clearly delineated
8	Tongue	Chemotherapy	Clearly delineated	Could not detect diffuse Ca. cells
9	Tongue	Chemotherapy	Mistake salivary gland as tumor Mistake adipose tissue as tumor Mistake scar tissue as tumor	Could not detect diffuse Ca. cells
10	Tongue	None	Mistake adipose tissue as tumor	Mistake adipose tissue as tumor
11	Tongue	None	Clearly delineated	Clearly delineated
12	Tongue	Interstitial Ra.	Clearly delineated	Clearly delineated
13	Oral floor	Chemotherapy	Mistake salivary tissue as tumor	Clearly delineated
14	Tongue	Interstitial Ra.	Mistake scar tissue as tumor	Clearly delineated
15	Tongue	Chemotherapy	Mistake adipose tissue as tumor	Clearly delineated
16	Tongue	Chemotherapy	Mistake adipose tissue as tumor Mistake scar tissue as tumor	Mistake adipose tissue as tumor
17	Tongue	None	Clearly delineated	Clearly delineated

Ca.: carcinoma, Ra.: radiotherapy

Figure 1


Radical resection of tumor
Vertical sectioning of surgical specimen
Examination without tissue staining
Examination with tissue staining
Histopathological examination

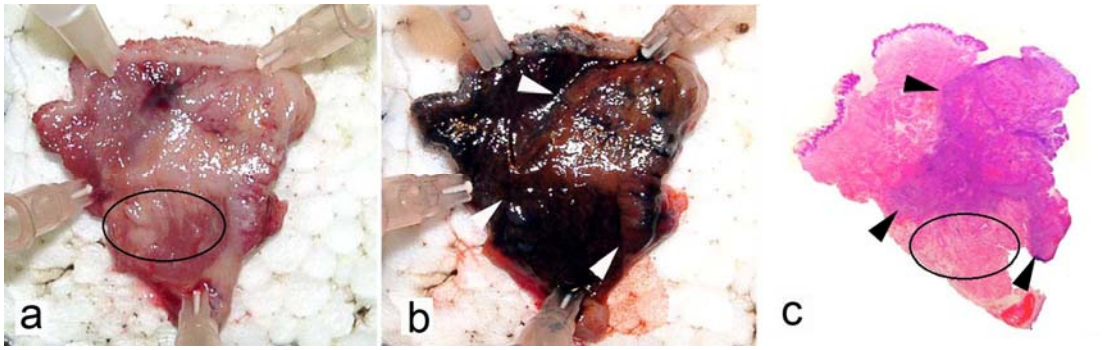


Figure2

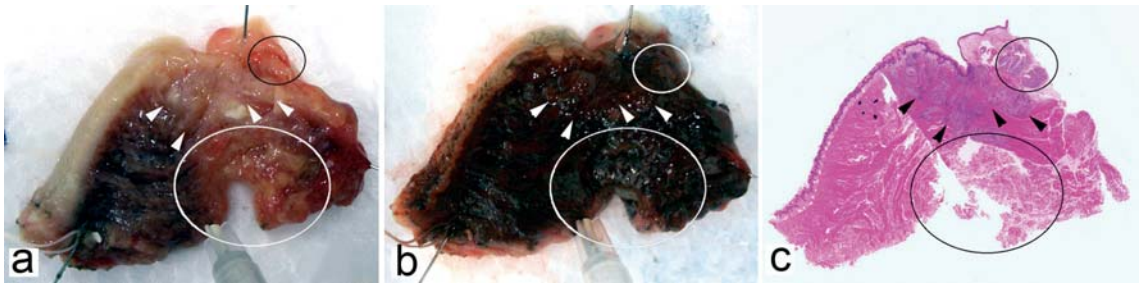


Figure3

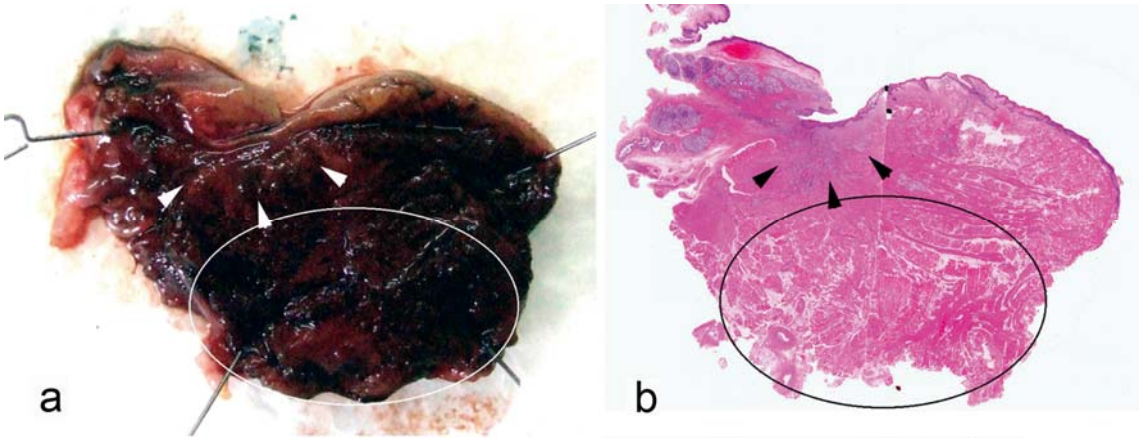


Figure4