

Histopathological evaluation including cytokeratin 13 and Ki-67 in the border between Lugol-stained and -unstained areas

KOUJI OHTA¹, IKUKO OGAWA³, SHIGEHIRO ONO¹, MASAACKI TAKI¹, KUNIKO MIZUTA¹, MIWA MIYAUCHI¹, MASAACKI TAKECHI¹, HIDEO SHIGEISHI¹, TAKASHI TAKATA² and NOBUYUKI KAMATA¹

¹Department of Oral and Maxillofacial Surgery, Division of Cervico-Gnathostomatology, Programs for Applied Biomedicine and

²Department of Oral and Maxillofacial Pathobiology, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University; ³Center of Oral Clinical Examination, Hiroshima University Hospital, Hiroshima, Japan

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Abstract. Lugol's iodine staining (Lugol-staining) has been widely used to detect malignant changes in the cervix uteri and esophagus. However, pathological and histochemical changes in the border between Lugol-stained and -unstained areas in oral epithelial dysplasia and malignant lesions are not well understood. We examined the histological appearance of 20 cases of epithelial dysplasia surrounding squamous cell carcinoma using HE and PAS staining in the borders between Lugol-stained and -unstained areas. Subsequently, differences in the expression of cytokeratin 13 (CK13), an epithelium differentiation marker, and Ki-67, a cell proliferative marker, in those areas were investigated by immunohistochemistry. Lugol-stained areas of all cases showed mild dysplasia or normal epithelium appearance, while Lugol-unstained areas in most cases were diagnosed as moderate/severe dysplasia and carcinoma *in situ*. PAS reactions were limited or not found in the Lugol-unstained areas as compared to intense positivity in Lugol-stained areas. CK13 and Ki-67 protein expression was significantly different between Lugol-stained and -unstained areas. It was confirmed that epithelia showing precancerous or cancerous features were detected as Lugol-unstained boundary areas. A reduction in glycogen production caused by alterations of cell differentiation and proliferation associated with malignant changes may result in a lack of Lugol-staining.

Introduction

Dysplastic changes, have a high potential for malignant transformation and, can be found in epithelium surrounding an oral squamous cell carcinoma (1,2). However, the region of

epithelial dysplasia is not be easily distinguished macroscopically from normal epithelium, thus Lugol's iodine staining (Lugol-staining) is widely used to detect malignant changes in the cervix uteri and esophagus (3,4). On the other hand, few studies have demonstrated the utility of Lugol-staining for detecting oral malignant lesions, and it has not been clarified whether Lugol-unstained areas surrounding oral dysplasia and a squamous cell carcinoma should be categorized as pre-malignant epithelia and resected.

Lugol-staining is linked to a reaction with glycogen in the cytoplasm (5). Malignant cervical lesions have a limited amount of glycogen as compared with normal squamous epithelium, thus they are revealed by a lack of Lugol-staining, whereas normal areas are dyed a brown-black color (6). However, changes related to cell differentiation and proliferation in the borders between Lugol-stained and -unstained areas in cases with oral dysplastic lesions are not well understood.

Keratins are considered to be excellent markers of keratinocyte differentiation and can reflect different patterns of keratinization. The expression of specific types, such as cytokeratin (CK)4 and CK13, is decreased in dysplastic lesions in the oral epithelium, and such changes have been associated with the development of oral dysplasia and squamous cell carcinomas (7,8). On the other hand, Ki-67 is a representative molecular marker of proliferating cells, and its labeling index correlates with the grade of dysplasia and severity of malignancy (9,10). In the present study, to elucidate the changes related to cell differentiation and proliferation in borders distinguished by Lugol-staining in and epithelial dysplasia surrounding squamous cell carcinoma, we investigated the histological appearance in the borders between Lugol-stained and -unstained areas using hematoxylin and eosin (HE) and periodic acid-Sciff (PAS)-staining. Subsequently, we examined the changes in CK13 and Ki-67 protein expression in those areas using immunohistochemistry.

Materials and methods

Twenty cases of oral epithelial dysplasia surrounding oral dysplasia and oral squamous cell carcinoma arising from

Correspondence to: Dr Kouji Ohta, Division of Cervico-Gnathostomatology, Department of Oral and Maxillofacial Surgery, Programs for Applied Biomedicine, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-Ku, Hiroshima 734-8553, Japan
E-mail: otkouji@hiroshima-u.ac.jp

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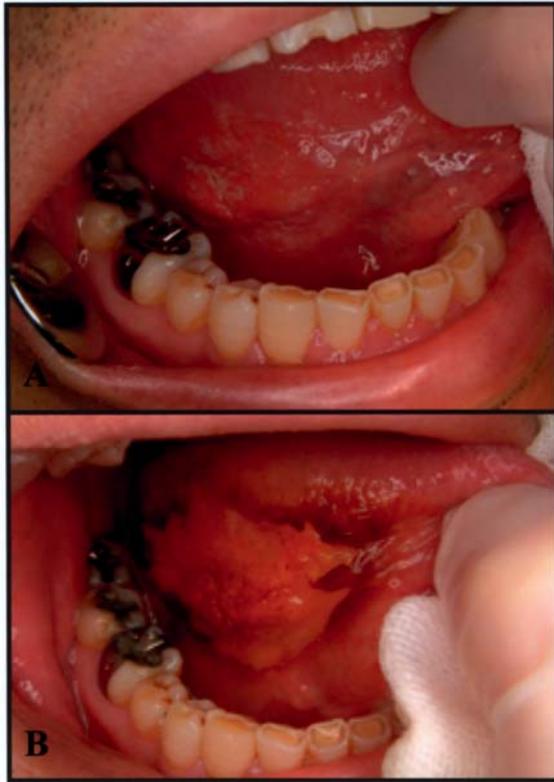


Figure 1. Oral epithelial dysplasia surrounding squamous cell carcinoma of the tongue. (A) Clinical appearance before Lugol-staining. (B) Appearance after Lugol-staining.

mucosa of the tongue, floor of the mouth or buccal mucosa, which were made up of a weakly parakeratinized epithelium, were examined in this study. Because a highly keratinized epithelium does not normally retain the Lugol solution. Lugol-staining was performed as follows. First, the lesion was rinsed with water and dried, then, it and surrounding area were subjected to Lugol-staining using a cotton swab before resection (Fig. 1). About 2 min after application, specimens from the boundary area between Lugol-stained and -unstained areas were obtained from 5 mm outside the boundary line, marked by an incision made along the boundary. Informed consent for the procedure was obtained from each patient according to a protocol approved by the Ethics Committee of Hiroshima University.

Light microscopy. The specimens were fixed in 10% formalin and embedded in paraffin, then sections $\sim 5 \mu\text{m}$ thick were cut and stained with HE and PAS. Two investigators confirmed the diagnosis of the specimens according to criteria proposed by the WHO.

Immunohistochemistry. Immunohistochemical staining of formalin-fixed, paraffin-embedded sections was done using an Envision kit (Dako, Glostrup, Denmark). After blocking endogenous peroxidase with methanol containing 0.3% H_2O_2 for 30 min, microwave pretreatment in citrate buffer (pH 6.0) was performed for antigen retrieval. The sections were incubated with the primary antibodies for CK13 and

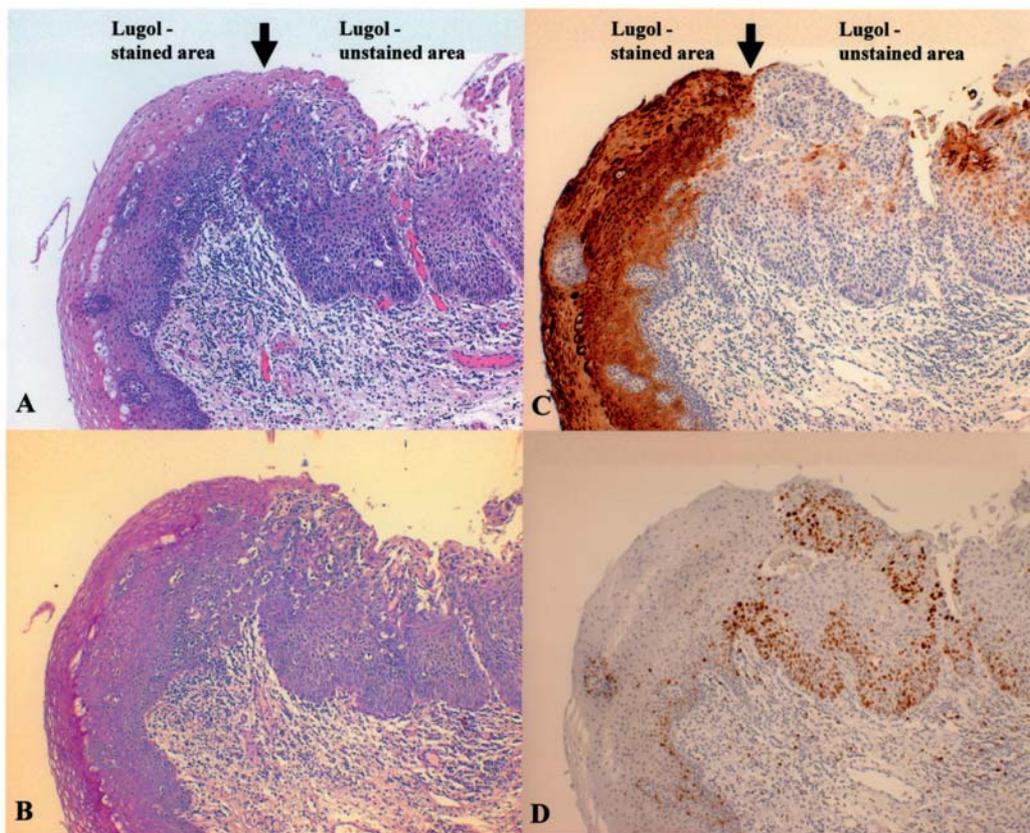


Figure 2. Histopathological and immunohistological findings in the border between Lugol-stained and -unstained areas in case 1. (A) HE staining, (B) PAS staining (C), CK13 immunostaining, (D) Ki-67 immunostaining. Small arrows indicate the border between Lugol-stained and unstained areas.



Case no.	Gender/Age	Location	Histological diagnosis ^a	
			Lugol-stained area	Lugol-unstained area
1	M/59	Tongue	Mild	Severe/CIS
2	F/77	Tongue	Normal, mild	Moderate, severe/CIS
3	F/68	Tongue	Normal, mild	Moderate
4	M/66	Tongue	Mild	Severe/CIS
5	F/80	Buccal mucosa	Normal, mild	Severe/CIS
6	M/83	Buccal mucosa	Normal	Moderate
7	M/56	Tongue	Normal	Moderate
8	F/80	Tongue	Normal, mild	Severe/CIS
9	F/73	Tongue	Normal	Mild, moderate
10	M/68	Tongue	Normal	Severe/CIS
11	M/74	Tongue	Normal	Mild, moderate
12	M/70	Mouth floor	Normal	Moderate
13	M/69	Tongue	Normal	Mild, moderate
14	M/44	Tongue	Normal	Moderate
15	M/58	Tongue	Mild	Severe/CIS
16	F/43	Tongue	Normal, mild	Moderate, severe/CIS
17	M/56	Tongue	Normal	Moderate, severe/CIS
18	M/65	Tongue	Mild	Severe/CIS
19	F/72	Tongue	Normal, mild	Moderate, severe/CIS
20	M/35	Tongue	Normal	Mild, moderate

^aMild, moderate and severe indicate the degree of dysplasia. CIS indicates carcinoma *in situ*.

Ki-67 (Dako, working dilutions 1/100 and 1/50, respectively) overnight at 4°C, followed by incubation with labeled polymer-peroxidase anti-mouse immunoglobulins for 30 min at room temperature. Color was developed with 3,3'-diaminobenzidine. Specificity was ascertained by substituting normal mouse IgG for the primary antibody. Two investigators evaluated the intensity of staining for CK13, which was classified as non-staining or sporadic (-), weak and/or patchy (+), strong and homogeneous (++) . Ki-67 labeling index was determined by the examination of at least 500 epithelial cells at x200 magnification in Lugol-stained or -unstained areas of each case and classified as 0-5% (+), 6-25% (++) , >25% (+++).

Results

Tissue samples from the border between Lugol-stained and -unstained areas contained mucous epithelia with various histopathological findings, including no obvious changes, various degrees of dysplasia, and carcinoma *in situ*. In all the cases stained by Lugol solution in the border areas, the mucous epithelium showed normal appearance or only slightly dysplastic changes (Table I). On the other hand, Lugol-unstained areas were histopathologically diagnosed as moderate to severe dysplasia or carcinoma *in situ* in 16 cases (Table I). In 12 of those cases, the demarcation between normal or mild dysplastic and highly dysplastic

epithelium including carcinoma *in situ*, was clear and easily identifiable in the HE-stained sections (Fig. 2A). In the other 4 cases, the degree of dysplasia gradually decreased and dysplastic changes such as abnormal variation in cell size, increased nuclear-cytoplasmic ratio, or increased nuclear size, spread to the basal/parabasal layers of the epithelium in the border area (Fig. 3A). In Lugol-stained areas, the surface of the epithelium showed regular parakeratosis, whereas Lugol-unstained areas were covered with highly or irregularly parakeratinized or orthokeratinized epithelium (Table II). Eight cases had an orthokeratinized layer in Lugol-unstained areas, of which 3 were consistent with cases histologically evaluated as having mild to moderate dysplasia in the border (Table II).

Lugol-stained areas corresponded with epithelia showing PAS-positive reactions. In these areas, a large number of PAS-positive glycogen granules were observed in the cytoplasm of epithelial cells on the surface and in prickle cell layers (Figs. 2B and 3B). In contrast, epithelial cells in Lugol-unstained areas had limited or no PAS reactions (Figs. 2B and 3B). Although negativity for PAS-staining was generally related to a high grade of epithelial dysplasia, including carcinoma *in situ*, in the border of Lugol-staining, the dividing line between the PAS-positive and -negative areas did not necessarily correspond with the border line between normal or mild dysplasia and moderate to severe dysplasia/carcinoma *in situ* observed in HE-stained sections. In 9 cases, the

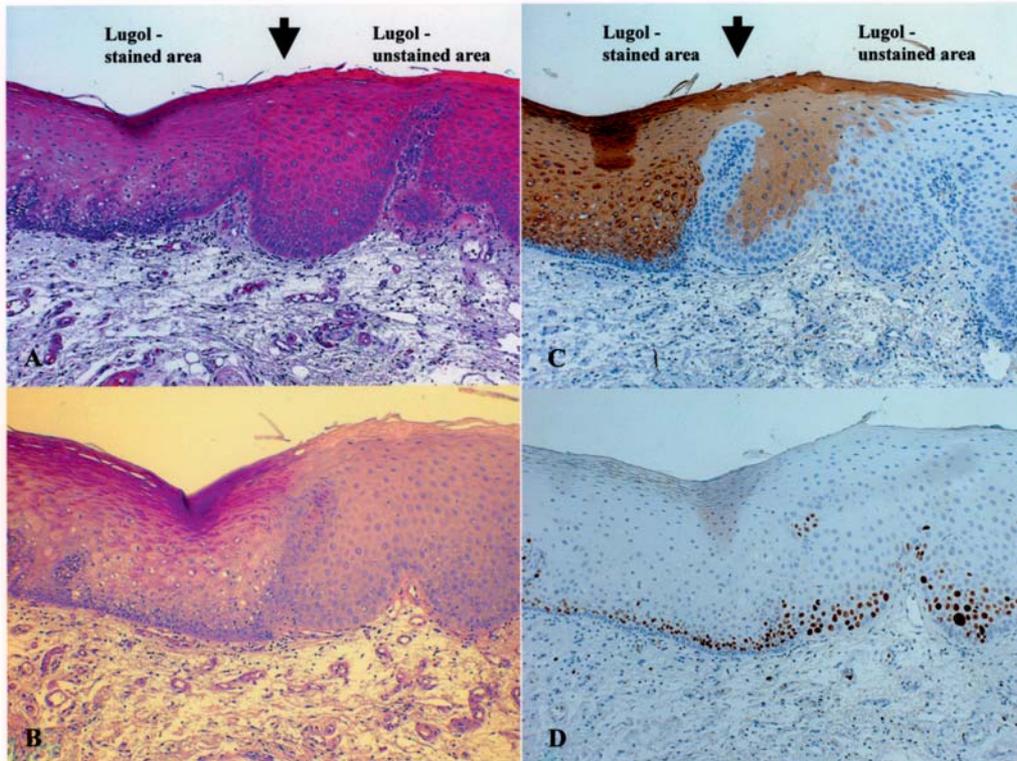


Figure 3. Histopathological and immunohistological findings in the border between Lugol-stained and -unstained areas in case 2. (A) HE staining, (B) PAS staining, (C) CK13 immunostaining (D) Ki-67 immunostaining. Small arrows indicate the border between Lugol-stained and -unstained areas.

Table II. Histological findings of the surface epithelium and expression of CK13 and Ki-67 protein in the border between Lugol-stained and -unstained areas.

Case no.	Lugol-stained area			Lugol-unstained area		
	Keratinization ^a	CK13 ^b	Ki-67 ^c	Keratinization ^a	CK13 ^b	Ki-67 ^c
1	PK	++	+	PK	+	+++
2	PK	++	+	PK/OK	-	++
3	PK	++	+	OK	+	++
4	PK	++	+	PK/OK	-	++
5	PK	++	+	PK	-	+++
6	PK	++	+	PK	+	++
7	PK	++	+	PK	+	++
8	PK	++	+	PK	+	+++
9	PK	++	+	OK	+	++
10	PK	++	+	PK	+	+++
11	PK	++	+	PK	+	++
12	PK	++	+	OK	+	++
13	PK	++	+	PK/OK	+	++
14	PK	++	+	PK	+	++
15	PK	++	+	PK	-	+++
16	PK	++	+	PK	+	++
17	PK	++	+	PK	+	++
18	PK	++	+	PK	-	+++
19	PK	++	+	PK/OK	+	+++
20	PK	++	+	OK	+	++

^aPK indicates parakeratosis. OK indicates orthokeratosis. ^bCK13 expression is indicated as non-staining or sporadic (-), weak and/or patchy (+), and strong and homogeneous (++) . ^cKi-67 expression is indicated as 0-5% (+), 6-25% (++) , and >25% (+++).



line of PAS-staining was identical to that of the pathological border evaluated in HE-stained sections, whereas it was located on the lesional side of the pathological border in 11. In those latter cases, a large number of PAS-positive granules was also present on the surface of the dysplastic epithelia. In addition, orthokeratinized areas were always negative for PAS-staining regardless of the degree of epithelial dysplasia.

CK13 and Ki-67 expressions were significantly different between Lugol-stained and -unstained areas (Table II). CK13 was regularly and highly expressed throughout the epithelium except for basal cells in Lugol-stained areas (Figs. 2C and 3C). Whereas, that expression was markedly reduced in Lugol-unstained areas, and sporadically or weakly expressed in the border (Figs. 2C and 3C). Ki-67 was detected in basal/parabasal cells of Lugol-stained areas, though it was more widely expressed in unstained areas (Figs. 2D and 3D). The changes in expressions of these two proteins were related to the pathological border observed in HE-stained sections.

Discussion

In the present study, we found that alterations of CK13 and Ki-67 protein expression between Lugol-stained and -unstained areas of oral dysplasia surrounding squamous cell carcinoma. CK13 is a differentiation-specific keratin, and Bloor *et al* reported that its protein expression was reduced in moderate and severe dysplasia (7). Furthermore, the level of CK13 mRNA expression in squamous cell carcinoma and severe dysplasia was reported to be lower than that in mild to moderate dysplasia (8). The present results showed that CK13 protein was intensely and regularly expressed in epithelia, except for basal cells in Lugol-stained areas, whereas it was markedly decreased in Lugol-unstained areas. The differences in expression clearly demonstrated the boundary line, which was consistent with the pathological border in HE-stained sections. Ki-67 protein is a representative molecular marker of proliferating cells that is extensively used in examination of oral epithelial dysplasia and squamous cell carcinoma (9,10). In the present study, Ki-67 protein expression was detected in the basal/parabasal layers of Lugol-stained areas, whereas Ki-67 positive cells were increased in Lugol-unstained areas and also found in supra-basal prickle cell layers. In the border between Lugol-stained and -unstained areas, differences in the number and localization of Ki-67-positive cells were observed. These findings correspond with previous findings showing that the total number of proliferating cells, which increases according to the degree of epithelial dysplasia and aggregation of Ki-67-positive cells in the suprabasal layer, is a useful marker of high-grade dysplasia (11). Our results obtained with the immunohistochemical staining for CK13 and Ki-67 confirmed the ability of Lugol-staining to demonstrate the precise border between low- and high-grade dysplasia. Differentiation and proliferation changes related to malignant transformation may occur in dysplastic epithelium in the border between Lugol-stained and -unstained areas.

Lugol-staining produces a brown-black color based on the reaction of iodine with glycogen granules (12). The tongue,

cheek and mouth floor areas are covered with slightly parakeratotic epithelium that contains glycogen granules in the cytoplasm of the prickle and surface cell layers, resulting in positivity for Lugol-staining. Glycogen content is inversely related to the degree of keratosis, suggesting a role of glycogen in keratinization (13). Malignant epithelium has a limited amount of glycogen granules as compared with normal squamous epithelium (3). It has been reported that intermediate filaments, precursors of keratosis, in epithelial cells are increased in the cytoplasm according to the degree of epithelial dysplasia, while glycogen granules in moderate and severe dysplasia are significantly decreased compared with those in the normal epithelium (14). In the present study, all border areas stained by Lugol solution were normal or mild dysplastic epithelium corresponded with PAS-positive, which indicates parakeratotic epithelium containing a large amount of glycogen. On the other hand, Lugol-unstained areas, which were PAS-negative, showed irregularly parakeratotic or orthokeratotic epithelium, in which the degree of dysplasia was higher and the amount of glycogen lower as compared to Lugol-stained areas. However, the dividing lines between the PAS-positive and -negative areas were not necessarily in agreement with the pathological borders. Kurita *et al* reported that wide differences were occasionally found between the dividing lines in dysplastic epithelia denoted by PAS staining and those by iodine staining, and noted that the branched structure of glycogen granules may break down in the process of degeneration, while the PAS-reactive character remained (15). Thus, glycogen granules in the border of the Lugol-unstained area may be in the process of degeneration. The correlation between CK13 expression and glycogen content suggests that loss of cellular differentiation may render the cells incapable of producing glycogen (16). Therefore, a reduction in glycogen production caused by alterations of cell differentiation and proliferation associated with malignant changes may result in the lack of Lugol-staining.

Some investigators examined whether the Lugol-unstained region surrounding an oral squamous cell carcinoma should be resected surgically together with the tumor. Yajima *et al* reported that telomerase activity was significantly different between iodine-stained areas of normal epithelium and -unstained areas of epithelial dysplasia (17). In another study, p53 expression in iodine-unstained areas was markedly higher than in iodine-stained areas (14). Our findings along with those suggest that the Lugol-unstained area is consistent with premalignant and malignant lesions in the oral mucosa, and should be completely removed if it surrounds dysplasia or a squamous cell carcinoma.

In our study, Lugol-staining was helpful to determine surgical resection margins for removal of oral dysplastic epithelium. However, application is limited to non-keratinized mucosa, such as that of the tongue, cheek and oral floor, while this method also can not detect subepithelial infiltrating tumors (15). Based on our results, slight dysplasia categorized as mild grade could not be distinguished from normal epithelium by Lugol-staining. Additional clinical studies and long-time follow-up results are needed to distinguish surgical resection margins from Lugol-unstained areas in oral epithelial dysplasia surrounding a squamous cell carcinoma.

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