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Research paper

# Difference in glycogen metabolism (glycogen synthesis and glycolysis) between normal and dysplastic/malignant oral epithelium

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ABSTRACT

*Background:* The purpose of this study was to investigate a difference in glycogen metabolism (glycogen synthesis and glycolysis) between the iodine stained (normal non-keartinized) and the unstained (dysplasctic/malignant) oral epithelium.

*Methods:* Twenty-one frozen tissue samples of iodine-stained and unstained mucosal tissue were obtained from 21 OSCC patients. Serial frozen sections were cut and examined with the hematoxylin-eosin and periodic acid-Schiff methods and immunohistochemical (IHC) staining for Ki67, P53, molecules associated with glycogenesis (i.e., glycogen synthase (GS) and phospho-glycogen synthase (PGS)), and molecules associated with glycogenolysis (i.e., glycogen phosphorylase isoenzyme BB (GPBB) examine the glycogen metabolism in OSCC. Additionally, *in vitro* study, the expression levels of GS and GPBB in the cultured cells were analyzed by immunofluorescent staining, Western blot analysis, and the real-time quantitative polymerase chain reaction (PCR).

*Results:* There was no significant difference in GS and PGS immunoactivity between iodine stained and unstained area. On the other hand, significantly greater GPBB immunoreactivity was observed in the basal and parabasal layers of iodine-unstained epithelium, where higher positivity for p53 and Ki67 was also showed. Additionally, western blot analysis, immunofluorescent staining, and real-time quantitative PCR revealed that the oral squamous cancer cells exhibited greater expression of GPBB than normal epithelial cells.

*Conclusions:* The results of this study showed that GPBB expression, which resulted in up-regulation of glycogenolysis, is enhanced in oral dysplastic/malignant epithelium compared with non-keartinized normal epithelium, in spite of the fact that glycogenesis continues in both of them. Premalignant and malignant epithelial cells consume greater quantities of energy due to their increased proliferation, and hence, exhaust their glycogen stores, which resulting in negative stain reaction with iodine solution.

## 1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral cavity (Jemal, Bray, & Center, 2011). During the treatment of OSCC, surgical margin status is an important prognostic factor for both recurrence and overall survival (Binahmed, Nason, & Abdoh, 2007 ; Kurita et al., 2016; Xiao, Kurita & Shimane, 2013).To ensure a complete tumor resection is achieved, vital staining with iodine solution is widely used to discriminate between the normal and the dysplastic/malignant oral epithelium (Chisholm et al., 1992; Epstein, Scully, & Spinelli, 1992; Kurita & Kurashina, 1996; Ohta, Ogawa, & Ono, 2010). It is thought that the iodine reacts with glycogen existed in the cytoplasm of oral epithelium (Xiao et al., 2013). Iodine solution is retained in normal non-keratinized squamous epithelium, but not in severely dysplastic or malignant epithelial tissue, because of differences in the glycogen content of the cytoplasm between them (Dawsey, Fleischer, & Wang, 1998; Nakanishi, Ochiai, & Shimoda, 1997; Ogawa, Washio & Takahashi, 2014; Shimizu, Tsukagoshi, & Fujita, 2001; Xiao et al., 2013;). The oral epithelium that exhibit dysplastic changes with a high potential for malignant transformation has been reported to contain reduced amounts of glycogen (Chisholm et al., 1992; Epstein et al., 1992; Xiao et al., 2013). It is well known that glucose consumption is highly elevated in oral dyplastic/malignant epithelial cells. Glycogen is thought to be “a store of glucose” and may also be used up. However, little is known about glycogen metabolism (glycogen synthesis and glycolysis) in oral dysplastic/

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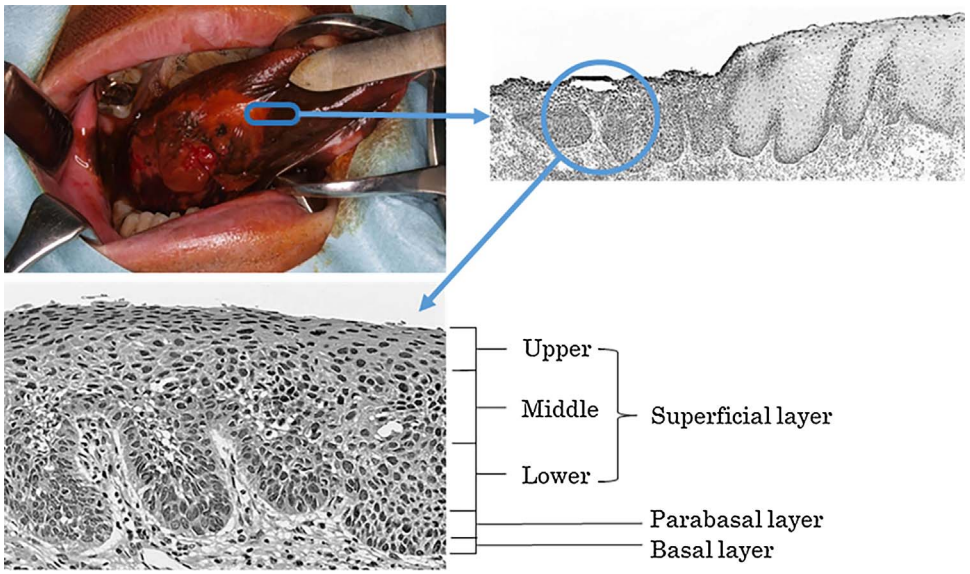


**Fig. 1.** Specimens collection.
(Left upper panel) A photograph of iodine vital staining of the tongue.
(Right upper panel) Specimens containing both iodine-stained and unstained mucosal tissue were obtained.
(Left lower panel) Various layers were stained with iodine solution and immunohistochemical techniques.

**Table 1**
Clinical and histopathological features and patients (n = 21).

| Characteristics | Number |
|---|---|
| Gender | |
| Male | 11 |
| Female | 10 |
| Age(years) | |
| Range | 26–92 |
| Median | 70 |
| Primary site | |
| Tongue | 12 |
| Floor of mouth | 1 |
| Gingival | 8 |
| Histopathological diagnosis | |
| Squamous cell carcinoma | 21 |

malignant epithelial cells (Ogawa et al., 2014; Xiao et al., 2013).

Therefore, the purpose of this study was to investigate a difference in glycogen metabolism (glycogen synthesis and glycolysis) between the iodine stained and the unstained oral epithelial tissue. We hypothesized that premalignant and malignant epithelial cells consume greater quantities of energy due to their increased proliferation, and hence, exhaust their glycogen stores.

## 2. Materials and methods

### 2.1. Patients

From June 2011 to September 2014, 55 consecutive patients with histologically proven primary OSCC were examined. Of these patients, 21 samples included both iodine stained and unstained area and were successfully subjected to frozen section analysis were included in this study. Written informed consent was obtained from all patients before their inclusion, and the study was approved by the ethics committee of the Shinshu University School of Medicine (No.1341).

### 2.2. Histology and immunohistochemistry

Dental iodine glycerin (10 mg iodine/100 ml; Showa Yakuhin, Japan) was used as the vital staining solution before the resection of the primary tumor. Iodine staining of the tissue around the lesion was performed as described by Kurita et al. (Kurita & Kurashina, 1996). Specimens that included both iodine-stained and unstained areas were obtained from the boundary region (Fig. 1). All specimens were stored at −80 °C after being snap-frozen in dry ice-cooled acetone (Xiao et al., 2013). Serial 5-μm frozen sections were cut and mounted on Matsunami adhesive silane-coated glass slides (Matsunami Glass, Japan). The first vital section was placed in xylene for 30 s after being subjected to 30 min air-drying at room temperature, and was then mounted onto a coverslip. These slides were subjected to light microscopic examinations and microphotography as soon as possible because the brown-black color of iodine can gradually disappear over the course of 2 weeks. The other sequential sections were subjected to hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS) staining. To ensure optimal cell structure preservation and that the tissue remained sensitive to immunostaining, 20% formalin neutral buffer solution was used to fix the sections, which were then subjected to 12 min of microwave treatment in Tris/ethylenediamine tetraacetic acid (pH 8.0) for antigen retrieval. Normal serum blocking and exclusion of the primary antibody were used to produce negative controls. Immunoreactivity was visualized with diaminobenzidine and hydrogen peroxide (Xiao et al., 2013). The antibodies used in this study included anti-p53 (malignant cell marker; 1:200 dilution; DO-7, Dako, Denmark), Ki67 (cell proliferation marker; 1:300 dilution; MIB-1, Dako, Denmark), glycogen synthase (GS) (glycogen synthesis marker; 1:100 dilution; 15B1, Cell Signaling Technology, Danvers, MA, USA), phospho-glycogen synthase (PGS) (glycogen synthesis marker; 1:100 dilution; Ser641, Cell Signaling Technology, Danvers, MA, USA), glycogen phosphorylase isoenzyme BB (GPBB) (glycolysis marker; 1:100 dilution; ab61036, Abcam, UK) and

**Table 2**
Specific primer for real-time PCR.

| *Gene* | *Sense primer* | *Antisense primer* |
|---|---|---|
| β-actin | 5′-GGACTTCGAGCAAGAGATGG-3′ | 5′-GTGGATGCCACAGGACTCCAT-3′ |
| GS | 5′-ATCGAGGCACAGCACTTG-3′ | 5′-GGCGATAAAGAAGTATAAGGTCTTG-3′ |
| GPBB | 5′-CCATCTATCAGTTGGGGTTAGACT-3′ | 5′-CTGCCAGCCATTGACAATC-3′ |