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Original article

# Brush biopsy of human oral mucosal epithelial cells as a quality control of the cell source for fabrication of transplantable epithelial cell sheets for regenerative medicine

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ABSTRACT

Autologous oral mucosal epithelial cell sheets have been used for treating epithelial defects such as cornea and esophagus. The cell source of patients' oral mucosal epithelial cell sheet should be examined in normality because it has individual difference. In this study, oral mucosal epithelial cells were less invasively collected by brush biopsy from the buccal, gingival, labial, and palate mucosa of four healthy volunteer donors without anesthesia, and analyzed the keratin expressions by western blotting and the obtained results were compared with those by immunohistochemistry of each of the native tissues. All of the oral mucosal epithelial cells expressed keratin 4 (K4) and K13, which were mucosal stratified squamous epithelial cell markers. K1 and K10, keratinized epithelial cell markers, were also detected in keratinized tissues such as gingival and palate mucosa. The markers of epithelial basal cells such as p63 and K15 were not detected by brush biopsy-western blotting. Although this method does not include basal layers of oral mucosa, protein expressions of upper layer of lesion area are different from normal. Therefore, brush biopsy-western blotting was extremely less invasive and would contribute to quality control of the fabrication of autologous oral mucosal epithelial cell sheets.


## 1. Introduction

We have successfully performed clinical applications of cell sheet based regenerative medicine in cornea [1] and esophagus [2] utilizing temperature-responsive cell culture surfaces [3,4]. Autologous oral mucosal epithelial cell sheets are used to promote wound healing and prevent stricture formation after the resection of superficial esophageal neoplasms by endoscopic submucosal dissection. This treatment is more effective than conventional treatments from the point of view of rapid wound healing and prevention of esophageal stenosis. According to the regulation for translational research by Japan's Pharmaceutical and Medical Devices Agency, transplantable cell sheets for the clinical use are fabricated to comply with the good-manufacturing-practice (GMP)-grade quality control (QC) for patient's safety [5]. The establishment of GMP-grade QC procedure requires assessing the management of manufacturing site, source, protocol, and products [6]. Especially, cell source of patient oral mucosal condition is often unstable due to individual difference. Therefore, the acquisition of the oral epithelial properties prior to fabricate cell sheets has an advantage in quality control [6,7]. Esophagus cancer occasionally coincides with oral cancer because the possible risk factors of both cancers are known to be smoking and alcohol intake [8,9], so when preparing autologous oral mucosal epithelial cell sheets, the oral cavity of patient should be carefully examined its property. For obtaining the source cells, the patient's oral biopsies were often performed after visual diagnosis. However, visual diagnosis is

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difficult to distinguish normal oral mucosa and premalignant lesion such as leukoplakia even by experienced doctor. Thus, sometimes, oral mucosal tissue has to be analyzed by immunohistology for definitive diagnosis. The protein expressions of premalignant lesion such as leukoplakia and oral lichen planus decreases K4 and K13 expressions and increases K1, K8, K10, K14, and K15 expressions in prickle cells of middle layer [10–16]. In addition, markers showing the abnormality of oral cavity are not only keratin expression but also epidermal growth factor receptor 4 (EGFR4) express in leukoplakia and alpha-defensin in candidiasis [17,18]. However, oral mucosa is resected as a cell source for cell sheets by spindle-shaped biopsy [19], and it is also resected as a specimen in order to examine oral mucosal epithelial cell properties. That is, implying that patient would be burdened by the twice biopsies. For reducing the patient's burden due to twice biopsies, therefore, we attempted to establish a simple test of apical oral mucosal cells which could analyze protein expressions. We focus on brush biopsy which could collect oral epithelial tissue with less invasiveness [20,21]. In this article, we examined combination of the brush biopsy and protein expression analysis of western blotting. Furthermore, we examined keratin expressions which representative epithelial marker by western blot analysis for investigative usefulness.

## 2. Methods

### 2.1. Preparation of samples for western blotting

This study was approved by the Institutional Review Board of Tokyo Women's Medical University, Tokyo, Japan. Oral mucosal epithelial cells and written informed consent was obtained from 4 healthy volunteers. Specimens were obtained from the buccal, gingival, labial, and palate mucosa of four healthy volunteers without smoking habit by dental brushes, which scraped the tissue 10 to 20 times with a brushed area of approximately 20 × 20 mm (400 $mm^2$) [Fig. 1]. Cells adhered to the bristles of brush were collected by agitating in 5.0 mL of Dulbecco's phosphate buffered saline (PBS) in a 50-mL centrifuge tube. The cells were transferred into a 15-mL centrifuge tube and centrifuged at 270 g for 5 min at 4 °C and the supernatant was removed from the tube. In addition, whole human oral epithelial tissue was collected at the time of a tooth extraction as a positive control lysate. The collected oral epithelial tissue was separated from connective tissue using dispase. These collected cells or tissue were solubilized with 237.5 μL Laemmli's sample buffer containing 12.5 μL 2-mercaptoethanol, and the solution was transferred into a 1.5-mL polypropylene micro tube [22]. For shearing DNA in the cells, the solution was sucked and pushed out 15 times through a 21-gauge needle attached to a 2.5-mL syringe, and sonicated for 3 min. The samples in the solution were denatured by heating for 5 min at 95 °C. The samples were centrifuged at 12000 g for 5 min at 4 °C and the supernatant was collected from the tube. The supernatant was stored at −80 °C until further use for western blotting analysis.

### 2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis

SDS-PAGE was performed by standard methods. The sample solution (10 μL) was loaded onto a polyacrylamide gradient gel (4–12% Bis-Tris Gel, Life technologies, Japan) and 2-(*N*-morpholino) ethanesulfonic acid buffer (running buffer; Life technologies). Electrophoresis was performed at 200 V and 120 mA for 40 min at room temperature with the running buffer. Separated protein bands were transferred to nitrocellulose membrane (Life technologies) for 6 min by a gel transfer device (Life technologies). After transfer, the membrane was blocked with 2% skim milk (membrane blocking agent, GE Healthcare, Japan) in tris buffered saline with tween 20 (TBS-T) for 1 h at 4 °C. For analyzing the transferred proteins, antibodies, which are shown in Table 1, were diluted in blocking buffer, and the membrane with antibody solution were incubated for overnight at 4 °C. The membrane was washed with TBS-T 6 times by the following time schedule 2, 2, 15, 5, 5, and 5 min, then incubated with peroxidase-labelled anti-mouse antibody or peroxidase-labelled anti-rabbit antibody (GE Healthcare) for 1 h at room temperature. After being washed with TBS-T 6

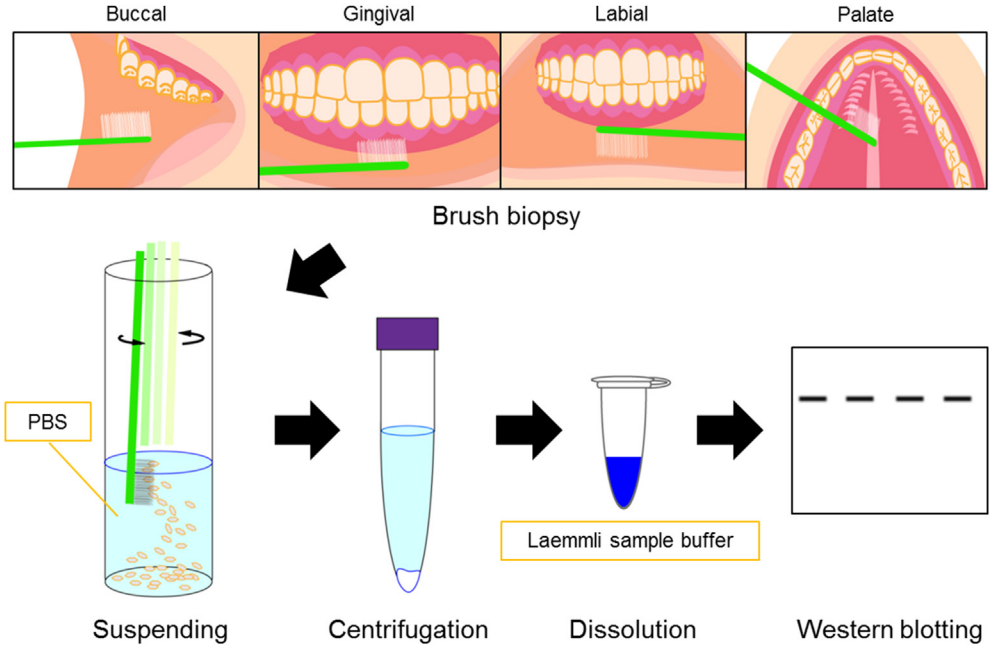


**Fig. 1.** Schematic diagram of the sampling method of brush biopsy. Samples were acquired from buccal mucosa, gingival mucosa, labial mucosa, and palate mucosa of oral cavity by using dental brushes. The dental brush was rotated for 10 to 20 revolutions. Collected cells were immediately agitated in PBS buffer. Cell suspension was centrifuged and supernatant was removed. Cells dissolved in Laemmli sample buffer.