Exfoliative Cytology of Oral Mucosa Epithelium： Cytochemical Study and Morphologic Analysis of Patients with Type 2 Diabetes

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Abstract

Background: Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia. An association between diabetes mellitus and periodontal disease has been reported. However, few comprehensive reports have examined the impact of chronic hyperglycemia on oral mucosal epithelial cells. This study clarified the influence of diabetes on oral mucosal epithelium cells.

Methods: Cells from 44 patients with type 2 diabetes and 10 healthy controls were collected from the buccal mucosa, gingiva, and lateral margin of the tongue by exfoliation. Samples were subjected to Papanicolaou staining, immunocytochemical staining using anti-AGE antibody, anti-Ki-67 antibody, and anti-p53 antibody, and lectin chemical staining. Furthermore, the nuclear area and cytoplasmic area were measured, and the nuclear/cytoplasm ratio was calculated.

Results: A chromatin condensation-like substance was found in samples from diabetic patients. In AGEs staining, positive cells were found in both groups, although the rate of positive cells tended to be higher in diabetic patients. No positive findings were found for Ki-67 and p53 antibodies. RCA_{120} and PNA showed differences in staining between diabetic patients and controls. The nuclear area was significantly expanded in diabetic patients ($P < 0.05$). There was no significant difference between groups in the

cytoplasmic area. The nuclear/cytoplasm ratio was significantly increased in diabetic patients ($P < 0.05$).

Conclusion: These findings suggest that in patients with persistent hyperglycemia, changes in the sugar chain terminal of the cell surface may influence mucosal epithelial cell differentiation. The appearance of chromatin-like substances may be an indicator of the need for better management of type 2 diabetes.

Keywords

Diabetes Mellitus, Oral Exfoliative Cytology, Cytochemistry, Lectins

1. Introduction

It is estimated that the total number of people with diabetes mellitus (DM) and incipient diabetes surpasses 20 million in Japan [1]. In addition, there are 420 million people with DM worldwide. It has been calculated that the worldwide population with DM will reach 700 million by 2025 if effective preventive measures are not taken [2]. DM is a metabolic disease characterized by chronic hyperglycemia in many patients; this hyperglycemia is associated with multiple complications [3]. Recently, several reports indicated that DM was associated with an increased risk for cancer [4] [5] [6] [7], including a 1.2 times increased risk for all cancers [8] [9], 1.85 times increased risk for pancreatic cancer [10], 2.5 times increased risk for liver cancer [11], and 1.30 times increased risk for colon cancer [12]. In terms of oral effects, it is reported that DM influences dry mouth, delay in healing, and periodontal disease [13] [14] [15] [16]. In addition, several researchers are conducting morphological searches for cells using exfoliative cytology, which is less invasive to patients, Seifi et al. [17] reported that the nuclear area and cytoplasmic area of oral mucosal epithelial cells in controls were significantly larger than in patients with DM. Jajarm et al. [18] reported that the nuclear and cytoplasmic areas, as well as the N/C ratio, were significantly larger in patients with DM than in controls. Alberti et al . [19] Shareef et al. [20], Sankhla et al. [21], Suvarna et al. [22], Sonawane et al. [23], and Sahu

et al. [24] reported that the nuclear area and N/C ratio of oral mucosal epithelial cells were significantly increased compared with control in patients with DM, but no significant differences in the area of the cytoplasm was seen.

However, few comprehensive reports have examined the effects of DM on mucosal epithelial cells, including cell proliferation and malignant transformation.

We have been conducting morphological studies on oral mucosa using exfoliative cytology and histochemical study method [25] [26] [27] [28].

In this study, we aimed to clarify the influence of type 2 DM on the oral mucosal epithelium by conducting cytochemical and morphologic research using exfoliative samples.

2. Subjects and Method

1) Subjects

A total of 44 patients (27 men and 17 women; mean age, 63.5 ± 15.0 years) with a diagnosis of type 2 DM for ≧ 1 year were included. They were diagnosed with internal medicine. Diagnostic criteria for type 2 diabetes were as follows: Plasma glucose levels (fasting ≧ 126 mg/dl, 2-hour OGTT ≧ 200 mg/dl, or casual ≧ 200 mg/dl) and HbA1c ≧ 6.5% [29]. These subjects were confirmed by medical history to be not DM by type 1 or other mechanisms.

The control group consisted of 10 healthy persons (5 men and 5 women; mean age 43.6 ± 13.7 years). All controls had fasting blood glucose and HbA1c NGSP (National Glycohemoglobin Standardization Program) values within the reference range.

Subjects with anemia, other systemic diseases, oral mucosal diseases, and smokers were excluded from the study.

All subjects had good oral cleaning status, as confirmed visually, and no noticeable inflammatory symptoms. Both DM patients and controls explained this experiment and received a signed consent form.

2) Cell collection method

The mouth was washed by tap water, and the absence of food residues in the oral

cavity was confirmed. Cells were collected from the gingival, buccal mucosa, and lateral margin of the tongue using a cell collection brush (Orcellex Brush®; Rovers Medical Devices Co., Ltd. Oss, The Netherlands). To obtain a smear, cell collection brush was rolled at least 5 to 10 times with gentle pressure.

3) Specimen preparation

Samples were prepared split for conventional cytology and liquid-based cytology (LBC) (TACAS™ method, MBL Co. Ltd., Aichi, Japan).

TACAS™ method details

Since formalin is not used for fixation, cell atrophy is mild, inflammatory cells, bacteria, necrotic cells, etc. A special slide glass is coated with a coating agent, and the surface is positively charged. Since the cells are negatively charged, they are smeared onto the glass by attracting each other. Since there is no smearing on the part without positive charge, the sample has less cell accumulation.

a) Conventional cytology

Oral mucosal epithelial cells were smeared on a glass slide and immersed in 95% alcohol for fixation.

b) LBC method

Oral mucosal epithelial cells were fixed for more than 30 min with fixative solution

(TACAS™ Amber, MBL Co., Ltd, Aichi, Japan) and then 1.5 mL per sample was transferred to a centrifuge tube and centrifuged at 2200 rpm for 5 min. The supernatant was removed and mixed with 3 mL distilled water (DW). Samples were centrifuged at 2200 rpm for 5 min, and the supernatant was removed again and mixed with 300 μL DW samples (300 μL) were placed on the slide, incubated for 10 min, and rinsed with 95% ethanol. Then they were performed immersionfixation in 95% ethanol.

4) Staining methods

a) Papanicolaou staining

Samples prepared using conventional cytology and the LBC method were rinsed with water, immersed in 0.5% hydrochloric acid alcohol 10 to 15 times, rinsed with 95% alcohol, and underwent OG-6 (Muto Pure Chemicals Co. Ltd. Tokyo, Japan) staining for 5 min. Samples were then rinsed again with 95% alcohol and underwent EA-50 (Muto Pure Chemicals Co., Ltd. Tokyo, Japan) staining for 5 min, were dehydrated in 95% and 100% alcohol, were cleared through xylene and then mounted on slides.

b) Immunocytochemical staining.

Slides prepared using the LBC method were rinsed with water. After blocking endogenous peroxidase activity with 3% H_2O_2-methanol for 15 min, each sample was rinsed with phosphate buffered saline (PBS) 3 times for 3 min. Anti-Ki-67

antibody(MIB-1, 1:50 dilution, Dako Cytomation, Glostrup, Denmark), anti-p53 antibody(DIO7, 1:50 dilution, Dako Cytomation, Glostrup, Denmark) , and anti-AGE antibody (6D12, 1:500 dilution, Trans Genic, Fukuoka, Japan),were used as primary antibodies.

AGE staining was done using the microwave method (0.01 M citric acid buffer solution pH 6.0, 500 W, 15 min); p53 staining was done using the pressure cooker method (Tris-EDTA buffer pH 9.0, 2 min heating); and ki-67 staining was done using a boiling process (Tris-EDTA buffer pH 9.0, 30 min), slow cooling, and rinsing each sample with PBS 3 times for 3 min.

Used the primary antibodies. After 30 min incubation at room temperature, each sample was rinsed with PBS 3 times for 3 min.

Samples were incubated at room temperature with a second antibody EnVision™ + Dual Link System-HRP (Dako Cytomation, Glostrup, Denmark) for 30 min and rinsed with PBS 3 times for 3 min.

Counter staining was performed with Carrazzi's Hematoxylin after color development in 3,3'-Diaminobenzidine, tetrahydrochloride (DAB) (Liquid DAB+ Substrate Chromogen System, Dako Cytomation, Glostrup, Denmark), rinsed with running water, and mounted on slides.

As a positive control, AGEs staining was used for diabetic kidney tissue, and Ki-67 and p53 staining was used for oral squamous cell carcinoma (OSCC) cases.

In AGEs immunocytochemical staining, 10 fields of view were observed with 400 × objective lens, and the ratio of positive cells to the observed cells of patients with DM and controls was calculated.

Judgment criteria

AGEs were positive if the cell surface and cytoplasm were stained brown, and Ki-67 and p53 were positive if the cell nucleus was stained brown.

c) Lectin cytochemical staining

Slide samples created using the LBC method were rinsed under running water. After blocking endogenous peroxidase activity with 3% H_2O_2-methanol for 15 min, samples were rinsed with PBS 3 times for 3 min. Lectin chemical study was examined by ConA, SBA, WGA, DBA, UEA-I, RCA_{120}, PNA (Biotinylated Lectin Kit I, Vecter, Burlingame, California, USA).

Incubated for 30 minutes at room temperature with Biotin lectin diluted by 20 μg/ml. After samples were rinsed with PBS 3 times for 3 min.

Incubated for 10 minutes at room temperature with Streptavidin/HRP (Dako Cytomation, Glostrup, Denmark) diluted by 2 μg/ml of EnVision™ + Dual Link System-

HRP (Dako Cytomation, Glostrup, Denmark). Samples were rinsed with PBS 3 times for 3 min. Counter staining with Carrazzi's Hematoxylin was performed after color development in DAB was done, and samples were rinsed DW and mounted on slides.

The evaluation of staining was performed using Image J (National Institutes of Health) to measure the brightness, and the following criteria were used based on 4 stages: (−) negative; (±) slightly positive; (+) moderately positive; and (++) strongly positive. Each luminance was defined as (−) ≧150; (±) 116 to 149; (+) 96 to 115; and (++) ≦ 95. Observation was performed by two dentists, and the average value was used.

5) Cytomorphologic analysis

Ten cells per field of view were extracted with 400 × magnification, and the cytoplasmic area and nuclear area were calculated with the Image J. The N/C ratio was calculated from these results.

For statistical analysis, the Mann-Whitney U test was used with Bonferroni's adjustment. All analyses were conducted with SPSS statistics, version 22 (IBM, Tokyo, Japan). A p value < 0.05 was considered significant.

The Nihon University School of Dentistry at Matsudo Ethics Committee approved this research (approval number: EC17-031).

3. Results

1) Papanicolaou staining

Of 44 samples from patients with DM, 23 showed dense hematoxylin stained substance in the nuclei of superficial cells. The patterns were rod-like, with incorrect margins. Substances connected to both poles in the nuclei (hereafter referred to as chromatin condensation-like substance (CCLS) are shown in (Figures 1(A)-(D)). These substances were found in both light green-positive and orange G-positive stained cells.

Cells with CCLS in the nucleus were found in about 0.8% of gingival specimens, with a breakdown of 0.6% for light green-positive cells and 0.2% for orange G-positive cells. Cells with CCLS in the nucleus were found in about 0.5% of buccal specimens, with a breakdown of 0.4% for light green-positive cells and 0.1% for orange G-positive cells. Cells with CCLS in the nucleus were found in about 0.3% of specimens from the lateral margin of tongue with a breakdown of 0.2% for light green-positive cells and 0.1% for orange G-positive cells. Figure 1(A) shows the high frequency of light-green stained cells in all areas. The frequency of appearance by area was gingival > buccal > lateral margin of the tongue. In the control group, only one CCLS cell was observed in 10 cases (Table 1).

2) Immunocytochemical staining

AGEs

Cytoplasm and cell membrane were positive in the samples from all 3 sites in patients with DM and controls. (Figures 2(A)-(F)).

The percentage of positive cells at each site was as follows: gingiva, 30% for patients with DM and 17% for controls; buccal muccosa, 32% for patients with DM and 21% for controls; lateral margin of tongue, 26% for patients with DM and 14% for controls. The proportion of positive cells tended to be higher in patients with DM than in controls.

Ki-67

No positive findings were found in the cell nuclei of patients with DM or controls. Positive reaction was detected of nuclear in OSCC.

p53

No positive findings were found in the cell nuclei of patients with DM or controls. Positive reaction was detected of nuclear in OSCC.

3) Lectin cytochemical staining

ConA

Negative or slightly positive findings were seen in the cytoplasm of patients with DM and controls.

SBA

Moderate to strongly positive findings were seen in the cytoplasm of patients with DM. Slight to moderately positive findings were seen in the cytoplasm of controls.

WGA

Moderate to strongly positive findings were seen in the cytoplasm of patients with DM and controls.

DBA

Negative findings were seen in the cytoplasm of patients with DM. Negative to moderately positive findings were seen in the cytoplasm of controls.

UEA-I

Negative to slightly positive findings were seen in the cytoplasm of patients with DM and controls.

RCA_{120}

Moderate to strongly positive findings were seen in the cytoplasm of patients with DM. Negative to moderately positive findings were seen in the cytoplasm of controls.

PNA

Moderate to strongly positive findings were seen in the cytoplasm of patients with DM. Slight to moderately positive findings were seen in the cytoplasm of controls.

PNA and RCA120 showed differences in lectin between patients with DM and

controls (Figures 3(A)-(D) and Table 2).

4) Cytomorphologic analysis

In terms of the nuclear area and the cytoplasmic areas, the nuclear area increased significantly in patients with DM ($p<0.05$). There were no significant differences between groups in the cytoplasmic area. The N/C ratio ($p<0.05$) was significantly increased in patients with DM. There were no significant differences between CCLS (+) and CCLS (−) (Table 3 and Figures 4-6).

4. Discussion

Papanicolaou staining showed that when cells of patients with DM were compared with cells of control subjects, CCLS was observed in the cell nuclei of patients with DM. It is well known that chromatin condensation is associated with cell proliferation [30]. Cell division can be observed only in the basal cell layer in the oral mucosal epithelium. Cells in the vicinity of basal cells cannot be collected by exfoliative cytology. In this study, CCLS were found in superficial cells. Based on this finding, it was suspected that these cells might be in the active proliferating stage.

Recently, the association of cancer and DM has been reported [8] [9] [10] [11] [12]. Therefore, it was necessary to examine the cell proliferating ability and malignant transformation ability of CCLS. Immunocytochemical studies were conducted using Ki-67, which is an indicator of cell proliferation ability, and p53, a tumor suppressor gene whose expression is observed during malignant transformation. Both Ki-67 and p53 studies showed negative findings. Based on these findings, the observed cells did not show an increase in cell proliferation ability or any malignant transformation. In addition, when cell division occurs, not only chromatin condensation, but also nuclear membrane loss occurs. The correlation with the cell cycle is considered to be low, considering the clearness of the nuclear membrane of cells with CCLS found in this study.

When the oral mucous membrane epithelial cells of patients with DM and controls were compared morphologically, the nuclear area and the N/C ratio was significantly larger in patients with DM compared to controls. The results of this study were similar to those of Alberti et al [19] Shareef et al. [20], Sankhla et al. [21], Suvarna et al. [22], Sonawane et al. [23], and Sa-hu et al. [24]. Nuclear swelling is commonly found in cells affected by the proliferation phase, inflammation, and malignant transformation. As a result of immunocytochemical staining in this study, there was no possibility of enhancement of cell proliferation ability or malignant transformation. In addition, because there were no clinical signs of inflammation in the oral cavity of the subjects, and the appearance of inflammatory cells was rarely observed in Papanicolaou-stained specimens of exfoliative cytology samples, it is assumed that this change was not attributable to inflammation.

The appearance of CCLS in the nucleus was mainly observed in the oral mucosal epithelial cells of patients with DM with a high HbA1c (NGSP) level (≥7.0%). It was assumed that the appearance of CCLS may be affected by sustained hyperglycemia. Therefore, we performed anti-AGE antibody in for samples of DM patients.

AGEs are produced when blood sugars, such as glucose, react with proteins (Maillard reaction). In DM, AGEs typical accumulate in the renal tube, retinas capillary, and blood

vessels walls. These AGEs cause an inflammatory response by inducing oxidative stress and increasing the expression of inflammatory cytokines.

It was reported that patients with DM had higher rates of AGEs-positive cells in the gingival epithelium compared to controls [31]. In this study, the incidence of AGEs-positive cells tended to be higher, although differences did not reach statistical significance in patients with DM in all parts of the gingiva, the buccal mucosa, and the tongue. These results were the same as the report by Zizzi et al. [32]. However, it cannot be denied that CCLS seen in this study may be due to increased oxidative stress caused by the accumulation of AGEs.

To investigate the influence of hyperglycemia, we further examined the binding of the sugar chain terminal using lectin cell chemistry. Lectins are proteins that bind to specific carbohydrate structures. Many kinds of lectins are isolated from plants, animals, and microorganisms. It is well known that changes in lectin binding patterns are related to cell differentiation and malignant transformation [33] [34]. In this study, 7 types of lectins, ConA, SBA, WGA, DBA, UEA-I, RCA120, and PNA, were used. A clear difference in staining was observed between patients with DM and controls in 2 types of lectins, RCA_{120} and PNA. This finding suggests that there was structure change in Galβ(1→3)-3GalNAc and D-Gal that sugar were chains on the oral mucosa epithelial cells of

patients with DM. As discussed above, sustained hyperglycemia is the most probable cause of this finding.

In Papanicolaou staining, CCLS appeared frequently in light-green cells in all sites. From this finding, it is considered that cells with weak keratinization tend to be susceptible to hyperglycemic effects. In addition, it is assumed that the cause of the abnormality in the nucleus was also continuous exposure to the hyperglycemic state in many patients, which promotes glycation and causes changes in the sugar chain tarminal of the oral mucosal epithelial cell membrane. These results have some influence on cell differentiation. The appearance of chromatin-like substances may be an indicator of the need for better management of type 2 diabetes.

The results observed in this study were suggested to be useful as a supplementary tool for determining the pathology of DM.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

References

1. Aya Morimoto, Rimei Nishimura, Naoko Tajima. (2010) Trends in the Epidemiology of Patients with Diabetes in Japan. JMA Journal, 53(1), 36–40.
2. World Health Organization. World Health Day 2016: Beat diabetes. (2016) https://www.who.int/campaigns/world-health-day/2016/en/.
3. Brajendra Kumar Tripathi, Arvind Kumar Srivastava. (2006) Diabetes mellitus: Complicationsand therapeutic.Medical Science Monitor, 12(7):RA130-47.
4. Kentaro Shikata, Toshiharu Ninomiya, Yutaka Kiyohara. (2012) Diabetes mellitus and cancer risk: Review of the epidemiological evidence. Cancer Science, 104, 9-14.
5. Gadi Shlomai, Brian Neel, Derek LeRoith, Emily Jane Gallagher. (2016) Type 2 DiabetesMellitus and Cancer: The Role of Pharmacotherapy. Journal of Clinical Oncology, 34, 4261-4269.
6. Shizuka Sasazuki, Hadrien Charvat, Azusa Hara, Kenji Wakai, Chisato Nagata. (2013) Diabetes mellitus and cancer risk: Pooled analysis of eight cohort studies in Japan. Cancer Science, 104, 1499–1507.
7. Masato Kasuga, Kohjiro Ueki, Naoko Tajima, Mitsuhiko Noda, Ken Ohashi. (2013) Report of the JDS/JCA Joint Committee on Diabetes and Cancer. Diabetology International, 4, 81-96.
8. Jessica L. Harding, Jonathan E. Shaw, Anna Peeters, Bendix Cartensen, Dianna J. Magliano. (2015) Cancer risk among people with type 1 and type 2 diabetes: disentangling true associations, detection bias, and reverse causation. Diabetes Care, 38, 264–270.
9. Manami Inoue, Motoki Iwasaki, Tetsuya Otani, Shizuka Sasazuki, Mitsuhiko Noda. (2006) Diabetes Mellitus and the Risk of Cancer: Results From a Large-Scale Population-Based Cohort Study in Japan. Archives of internal medicine, 166(17), 1871-1877
10. R Huxley, A Ansary-Moghaddam, A Berrington de Gonzalez, F Barzi, M Woodward. (2005) Type-II diabetes and pancreatic cancer: a meta-analysis of 36 studies. British Journal of Cancer, 92, 2076-2083.
11. El-Serag HB, Hampel H, Javadi F. (2006) The association between diabetes and hepatocellular Carcinoma: a systematic reviw of epidemiologic evidence. Clinical Gastroenterology and Hepatology, 4, 369-380.
12. Susanna C. Larsson, Nicola Orsini, Alicja Wolk. (2005) Diabetes Mellitus and Risk of Colorectal Cancer: A Meta-Analysis. Journal of the National Cancer Institute, 97, 1679-1687.

13. Peter Stanko, Lydie Izakovicova Holla. (2014) Bidirectional association between diabetes mellitus and inflammatory periodontal disease. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 158(1), 35-38.
14. Nilo Guliberto Martins Chávarry, Mario Vianna Vettore, Carmelo Sansone, AubreySheiham. (2009) The Relationship Between Diabetes Mellitus and Destructive Periodontal Disease: A Meta-Analysis. Oral Health and Preventive Dentistry, 7, 107-127.
15. P. M. Preshaw, A. L. Alba, D. Herrera, S. Jepsen, A. Konstantinidis. (2012) Periodontitis and diabetes: a two-way relationship. Diabetologia, 55, 21–31.
16. Fumi Seshima, Makiko Nishina, Takashi Namba, Atsushi Saito. (2016) Periodontal Regenerative Therapy in Patient with Chronic Periodontitis and Type 2 Diabetes Mellitus: A case Report. The Bulletin of Tokyo Dental College, 57(2), 97-104.
17. Safoura Seifi, Farideh Feizi, Zoleikhah Moazzezi, Mohammad Mehdizadeh, Babak Zamaniet. (2014) Evaluation of oral mucosal epithelium in diabetic male patients by exfoliative cytology method. Journal of Diabetes & Metabolic Disorders, 13, 77.
18. Hassan Hosseinpour Jajarm, Nooshin Mohtasham, Afsaneh Rangiani. (2008) Evalua-tion of oral mucosa epithelium in type II diabetic patients by an exfoliative cytology method. Journal of Oral Science, 50, 335-340.
19. Sandra Alberti, Cesar T. Spadella, Telma R. C. G. Francischone, Gerson F. Assis, Tania M. Cestari. (2003) Exfoliative cytology of the oral mucosa in type II diabetic patients: morphology and cytomorphometry. Journal of Oral Pathology & Medicine, 32, 538-543.
20. Ban Tawfeek Shareef, Kok Teong Ang, Venkatesh Ramasamy Naik. (2008) Qualitative and quantitative exfoliative cytology of normal oral mucosa in type 2 diabetic patients. Med Oral Pathol Oral Cir Bucal, 13(11), E693-6.
21. Bharat Sankhla, Sonali Shah, Madhusudhan AS, Priya Bhagatet. (2010) Exfoliative cytology of oral mucosa: Cytomorphometric analysis of diabetic patients. Journal of Oral Science, 2, 4-12.
22. M Suvarna, C Anuradha, K Kiran Kumar, P Chandra Sekhar, K Lalith Prakash Chandra, BV Ramana Reddyet. (2012) Cytomorphometric analysis of exfoliative buccal cells in type II diabetic patients. Journal of Dr. NTR University of Health Sciences, 1, 33-37.
23. Kishore Sonawane, Sandeep Jain, Indra Gupta, Karthik B.V., Sasidhar Singaraju, Medhini Singaraju. (2011) Cytomorphometric Analysis of Oral Mucosa in Diabetic Patients in Bhopal Region an In-Situ Study. International Journal of Clinical Dental Science, 2, 12-15.
24. Manisha Sahu, Hema Suryawanshi, Sushruth Nayak, Pramod Kumar. (2017) Cytomorphometric analysis of gingival epithelium and buccal mucosa cells in type 2 diabe-tes mellitus patients. Journal of Oral and Maxillofacial Pathology, 21(2), 224-228.

25. Yan Sun, Kayo Kuyama, Masahiko Fukumoto, Hitoshi Nishimura, Arne Burkhardt, Hirotsugu Yamamoto. (2011) The sequential changes of carcinoma cuniculatum:a case report with 11-year follow-up. Journal of Oral Pathology & Medicine, 15, 113-116.
26. Kayo Kuyama, Takashi Matsumoto, Miyuki Morikawa, Akira Fukatsu, Mana Ichimura, Masanobu Wakami, Masahiko Fukumoto, Taku Kato, Hirotsugu Yamamoto. (2013) Morphometrical findings among dysplasias of oral, cervical and bronchial regions. Open Journal of Stomatology, 13, 215-222.
27. Akira Fukatsu, Kayo Kuyama, Mana Fuchigami, Masahiko Fukumoto. (2015) Comparison of Liquid Based Cytology and Conventional Cytology in Squamous Cell Carcinoma. Journal of the Japan Academy of Dental Human Dock, 10, 41-48.
28. Fuchigami, M,Suemitsu, M,Fukatsu, A,Komine, C,Konish,i Y,Morikawa, M,Wakami M,Kuyama, K,Fukumoto M. (2016) Neutrophil elastate,CD68,HDL-DR and CD105 immunohistochemical expression of oral verruciform xanthoma. Journal of Japanease Society for Evidence and the Dental Professional, 8, 16-21.
29. Yutaka Seino, Kishio Nanjo, Naoko Tajima, Takashi Kadowaki, Atsunori Kashiwagi, Eiichi Araki. (2010) Report of the Committee on the classification and diagnostic criteria of diabetes mellitus The Committee of the Japan Diabetes Society on the diagnostic criteria of diabetes mellitus. Diabetology International, 1, 2-20.
30. Wolfram Antonin, Heinz Neumann. (2016) Chromosome condensation and decondensation during mitosis. Current Opinion in Cell Biology, 40, 15-22.
31. Schmidt AM, Weidman E, Lalla E, Yan SD, Hori O, Cao R, Brett JG, Lamster IB. (1996) Advanced glycation endproducts (AGEs) induce oxidant stress in the gingiva: a potential mechanism underlying accelerated periodontal disease associated with diabetes. Journal of Periodontal Research, 31, 508-515.
32. A. Zizzi, G. Tirabassi, S. D. Aspriello, M. Piemontese, C. Rubini, G. Lucarini. (2013) Gin-gival advanced glycation end-products in diabetes mellitus-associated chronic periodontitis: an immunohistochemical study. Journal of Periodontal Research, 48, 293-301.
33. Darcy A. Duncan, Michael T. Mazur, J. Benjamin Younger. Glycoconjugate distribution in normal human endometrium: A lectin histochemical study. International Journal of Gynecological Pathology, 7(3), 236-248.
34. Silvia Bopp, EI-Sayed EI-Hifnawi, Horst Laqua. (1992) Lectin binding pattern in human retinal pigment epithelium. Annals of Anatomy, 174(4), 279-285.

Figure

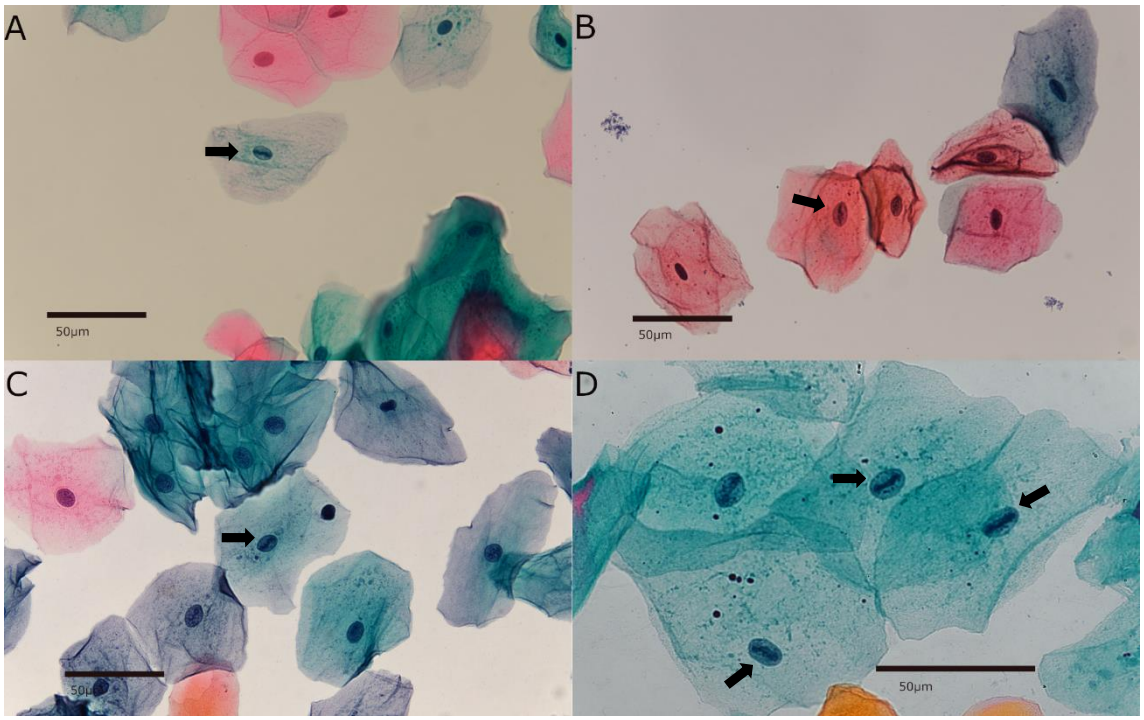

Figure 1. Papanicolaou staining. (A): buccal mucosa, (B): gingival, (C): lateral margin of the tongue, (D): gingival. CCLS is finding in the cell nucleus of each sample (arrow) ((A)-(D)). CCLS tends to be more in light green staining cells. CCLS looks like chromatin is condensation. Magnification: 60× ((A)-(C)), 100× (D). Scale bars represent 50 μm.

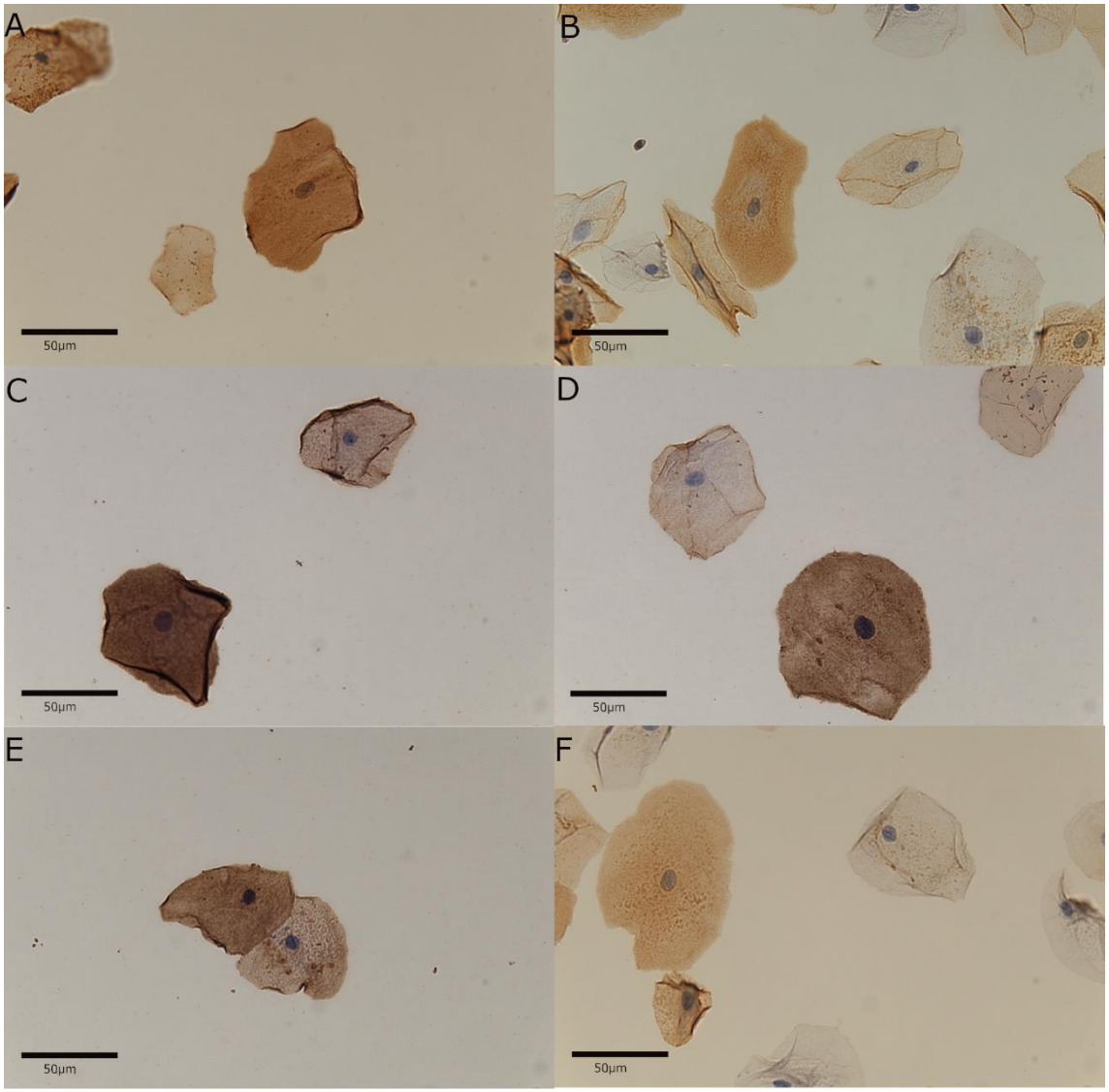

Figure 2. Immunocytochemical staining with AGE. (A): DM gingival, (B): control gingival, (C): DM buccal mucosa, (D): control buccal mucosa, (E): DM lateral margin of the tongue, (F): control lateral margin of the tongue. Positive reactions were observed in the cytoplasm and cell membrane of the specimens of DM patients and control (A)-(F). There are more positive cells in DM compared to control. Magnification: 60× (A)-(F). Scale bars represent 50 μm.

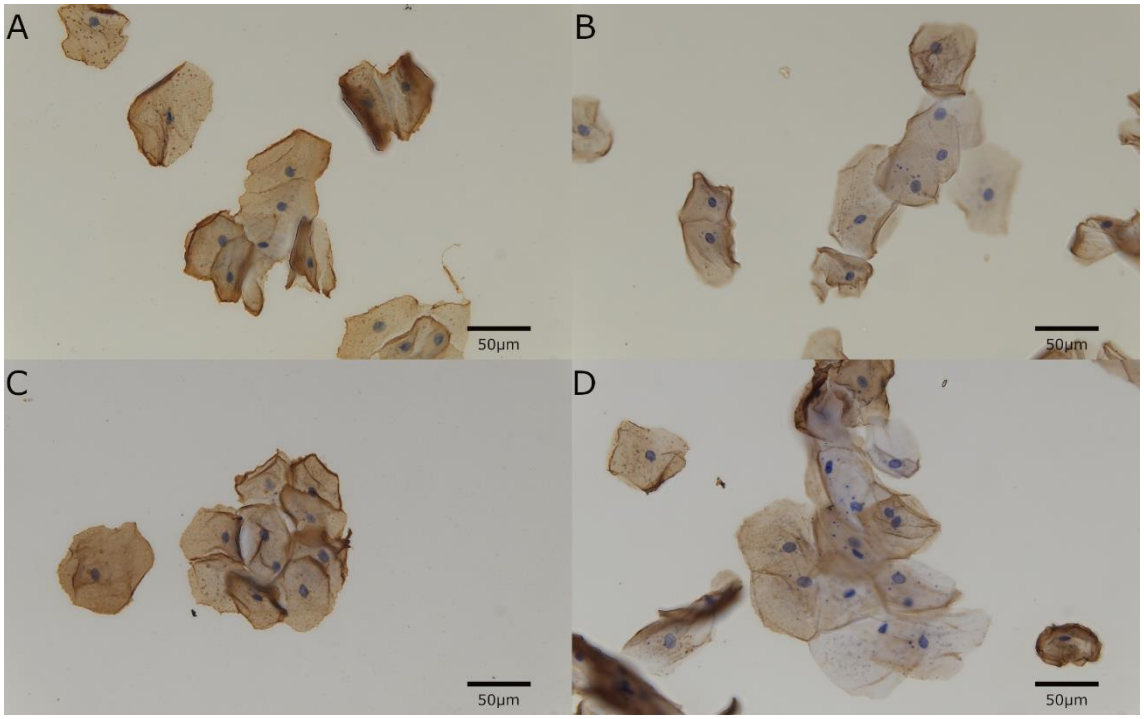

Figure 3. Lectin cytochemical staining. (A): DM RCA_{120}, (B): control RCA_{120}, (C): DM PNA, (D): control PNA. RCA_{120} positive reaction was detected ((A), (B)). PNA positive reaction was detected ((C), (D)). There was a difference in staining intensity in both stains, DM showed more staining. Magnification: 40 × ((A)-(D)). Scale bars represent 50 μm.

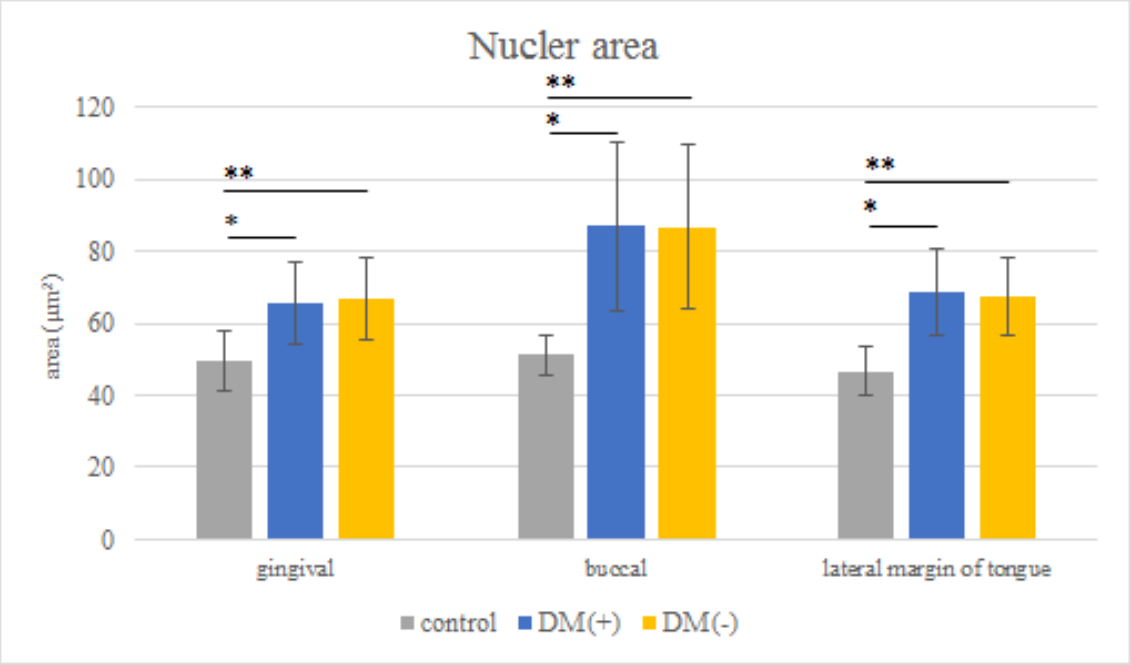

Figure 4. Comparison of nuclear area between DM (+), DM (−), and control. *p < 0.05 (vs DM (+)), **p < 0.05 (vs DM (-)). DM (+): CCLS, DM (-): CCLS.

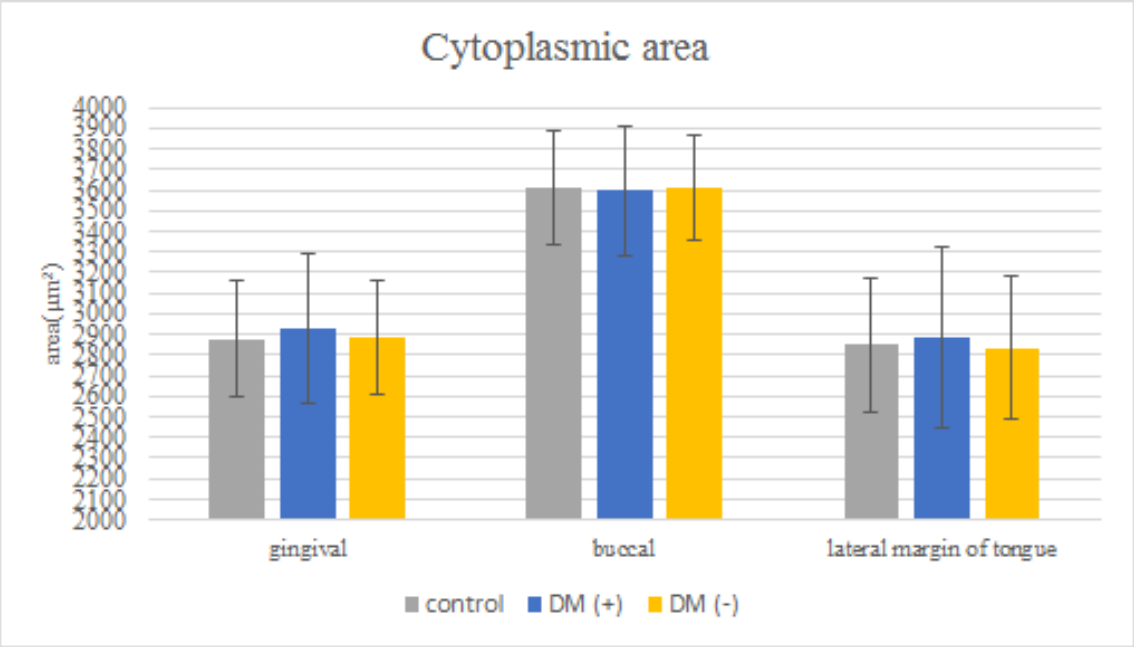

Figure 5. Comparison of cytoplasmic area between DM (+), DM (−), and control.

NS $p < 0.05$.

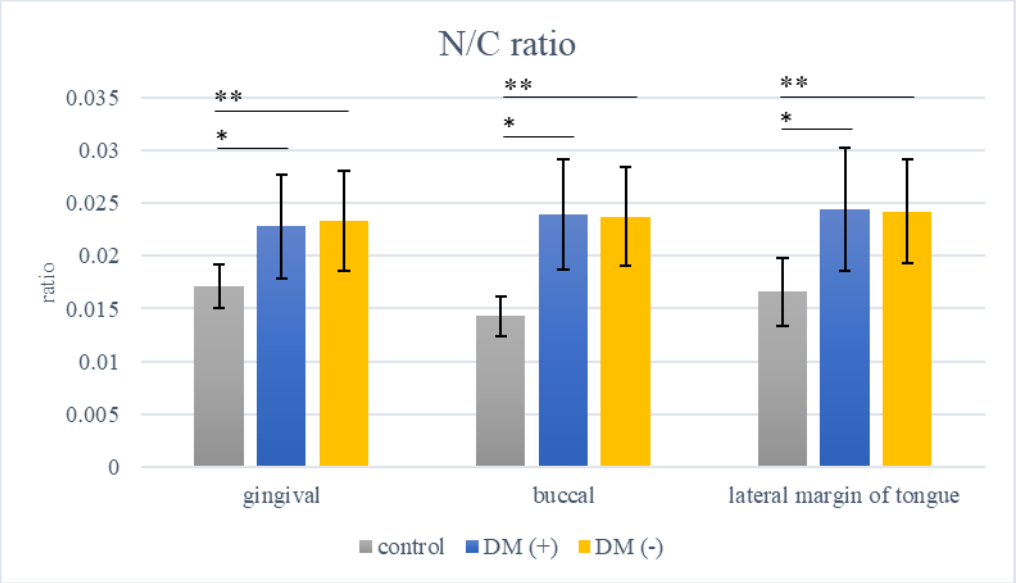

Figure 6. Comparison of N/C ratio between DM (+), DM (−), and control.

*$p<0.05$ (vs DM (+)), **$p < 0.05$ (vs DM (−)).

Table

Table 1. Papanicolaou staining results in patients with type 2 diabetes mellitus

No.	Buccal mucosa	Gingival	Tongue	HbA1c % (NGSP)	No.	Buccal mucosa	Gingival	Tongue	HbA1c % (NGSP)
1	+	+	+	11.8	23	+	-	+	7.2
2	+	+	+	11.3	24	-	-	-	7.1
3	+	+	+	10.4	25	-	-	-	7.1
4	+	+	+	10.2	26	+	+	+	7.1
5	+	+	+	9.1	27	-	-	-	6.9
6	+	-	+	9.1	28	-	-	-	6.9
7	+	+	-	9.0	29	-	-	-	6.9
8	+	+	+	8.7	30	-	+	-	6.9
9	-	+	+	8.7	31	-	-	-	6.7
10	+	+	+	8.3	32	-	-	-	6.5
11	+	+	+	8.0	33	-	-	-	6.4
12	+	+	+	7.9	34	-	-	-	6.4
13	+	-	-	7.8	35	-	-	-	6.3
14	-	-	-	7.7	36	-	-	-	6.3
15	+	+	+	7.7	37	-	-	-	6.1
16	+	+	+	7.6	38	+	+	+	6.1
17	+	+	+	7.6	39	-	-	-	5.9
18	+	+	+	7.5	40	-	-	-	5.9
19	+	+	+	7.4	41	-	-	-	5.8
20	+	+	+	7.3	42	-	-	-	5.8
21	-	-	-	7.3	43	-	-	-	5.6
22	-	-	-	7.3	44	-	-	-	5.2

+ : Chromatin with condensation-like substance; – : Chromatin without condensation-like substance.

Tongue= lateral margin of tongue. NGSP(National Glycohemoglobin Standardization Program)

Table 2. Lectin staining results

Lectin	Patients with Type 2 Diabetes	Controls
ConA	- to ±	- to ±
SBA	+ to ++	± to +
WGA	+ to ++	+ to ++
DBA	-	- to +
UEA-1	- to ±	- to ±
RCA_{120}	+ to ++	- to +
PNA	+ to ++	± to +

(-), negative; (±), slightly positive; (+), moderately positive; (++), strongly positive

Table 3. Cytomorphologic results

Detemination		DM(+) Mean ± SD	DM(-) Mean ± SD	Control Group Mean ± SD
Nucler area(μm^2)	gingival	65.68±11.28	66.58±11.44	49.34±8.42
	buccal	86.84±23.65	86.59±22.83	51.18±5.70
	tongue	68.60±12.27	67.51±10.59	46.70±6.80
Cytoplasmic area(μm^2)	gingival	2926.49±366.33	2882.70±277.53	2878.35±281.72
	buccal	3596.45±312.48	3612.72±252.61	3609.48±277.05
	tongue	2885.58±439.82	2832.53±348.29	2848.38±325.69
N/C ratio	gingival	0.0228±0.0049	0.0233±0.0047	0.0171±0.0021
	buccal	0.0239±0.0052	0.0237±0.0047	0.0143±0.0019
	tongue	0.0244±0.0058	0.0242±0.0049	0.0166±0.0032

DM(+)：Chromatin condensation-like substance having, DM(－)：Chromatin condensation-like substance not having

tongue：lateral margin of tongue