Differential Diagnosis of Well-Differentiated Squamous Cell Carcinoma from Non-Neoplastic Oral Mucosal Lesions: New Cytopathologic Evaluation Method Dependent on Keratinization-Related Parameters but Not Nuclear Atypism

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Background: The cytology of oral squamous cell carcinoma (SCC) is challenging because oral SCC cells tend to be well differentiated and lack nuclear atypia, often resulting in a false negative diagnosis. The purpose of this study was to establish practical cytopathological parameters specific to oral SCCs.

Methods: We reviewed 123 cases of malignancy and 53 of non-neoplastic lesions of the oral mucosa, which had been diagnosed using both cytology and histopathology specimens. From those, we selected 12 SCC and 4 CIS cases that had initially been categorized as NILM to ASC-H with the Bethesda system, as well as 4 non-neoplastic samples categorized as LSIL or ASC-H as controls, and compared their characteristic findings. After careful examinations, we highlighted five cytopathological parameters, as described in Results. Those 20 cytology samples were then reevaluated by 4 independent examiners using the Bethesda system as well as the 5 parameters.

Results: Five cytological features, (i) concentric arrangement of orangeophilic cells (indicating keratin pearls), (ii) large number of orangeophilic cells, (iii) bizarre-shaped orangeophilic cells without nuclear atypia, (iv) keratoglobules, and (v) uneven filamentous cytoplasm, were found to be significant parameters. All malignant cases contained at least one of those parameters, while none were observed in the four non-neoplastic cases with nuclear atypia. In reevaluations, the Bethesda system did not help the screeners distinguish oral SCCs from non-neoplastic lesions, while use of the five parameters enabled them to make a diagnosis of SCC.

Conclusion: Recognition of the present five parameters is useful for oral SCC cytology.

Key Words: concentric cellular arrangement; filamentous cytoplasm; keratinization; keratoglobules; oral squamous cell carcinoma
Oral cancer is one of the most common types of human cancers, ranking eighth among whole body cancers worldwide, and its frequency of oral cancer is well known to be higher in southern Asia, especially in developing countries, such as India and Pakistan. According to our own studies, it was sixth most common in Myanmar and the most prominent (19.6%) in Yemen. At the same time, increasing oral cancer frequency has been reported in European countries, including Denmark, France, Germany, and Scotland, with prevalence mainly in males. Since the most common type of oral cancer is squamous cell carcinoma (SCC), these statistics are considered to also reflect those for SCC.

Death and incidence rates of oral cancer according to age tend to be opposite in the United States as compared to Asian countries. In the United States, the age-adjusted death and incidence rates for males were decreased by 0.6- and 0.8-folds, respectively, in the 37 years up to 2012. In contrast, those rates in Taiwan were increased by 8.5- and 14.4-folds, respectively, in the 33-year period up to 2012. As for Japan, the age-adjusted death rate from oral and pharyngeal cancer was reported to have increased by 2.1-folds, while the rough overall rate increased by 8.5-folds in the past 57–62 years up to 2014. Such an obvious decreasing tendency of oral cancer in the United States seems to be the result of an early medical examination campaign for oral cancer prevention that features early detection and early treatment, for which cytology has been applied.

When compared with cervical and esophageal SCCs, oral SCCs show greater differentiation in histological findings, a phenomenon that may be due to differences in their embryonal origin, ectodermal or endodermal. Well-differentiated SCCs present one of the most challenging tasks for interpreting cytology findings of oral mucosal lesions, since they lack nuclear atypia, as described in the WHO Blue Book. However, cytological diagnosis is basically performed by referring to nuclear atypia appearing in any organ. Thus, when this cytological concept is applied to oral mucosal lesions, false-negative results are inevitable in significant numbers of oral SCC cases, as the sensitivity (true positive/true positive plus false-negative) of cytological diagnosis for oral cancer has been reported to vary at 87.4, 96.5, and 85.4%. These findings may indicate some limits of exfoliative cytology, although the reason for the higher rate of false-negative results has not been investigated in detail. Rather, in clinical practice, there is a general impression among clinicians that cytological diagnosis is not always reliable, especially in clinically gray-zone cases. Thus, it is important to establish oral-SCC-specific diagnostic criteria based on the concept that most oral SCC cases can be well differentiated and occasionally lack nuclear atypia, and that the Bethesda System widely used for clinical cytological examinations is primarily for cervical and not oral lesions.

The purpose of this study was to elucidate which cytological parameters other than nuclear atypia are effective for differential diagnosis of well-differentiated oral SCCs and non-neoplastic lesions with nuclear atypia. To this end, cytological findings were carefully compared with histological findings in oral carcinoma in situ (CIS) and SCC cases, which were objectively diagnosed based on immunohistochemistry findings.

Materials and Methods

Specimens
Cytology specimens were obtained from patients who visited the Oral Surgery Clinic of Yamanashi Prefectural Central Hospital, Kofu, Japan, during the 96-month period from 2004 to 2011. We examined a total of 603 specimens, which accounted for 0.82% of the 73,736 cytology specimens collected at that hospital during the same period. They were composed of 424 (70.3%) curette-scraping samples from oral mucosal surfaces and 179 (29.7%) fine needle aspiration samples from deeper soft parts, including lymph nodes and salivary glands. Initial screening of those 424 oral mucosal samples by curette scraping was randomly and independently performed by 4 screeners, each of whom had >10 years of experience in cytopathology (1 expert cytologist, 3 pathologists), to select specimens from non-neoplastic cases, which were determined by whether they contained >200 squamous epithelial cells smeared on a slide glass. Malignant cases were chosen when 30 or more diagnosable cells were present in the smear. From those, we then selected 123 cases of malignancy (119 SCCs, 4 CISs) and 53 cases with non-neoplastic lesions, both of which also had histology specimens in adequate condition available for re-evaluation using immunohistochemical hallmarks for oral malignancy, such as loss of keratin (K) 13 or K19, as well as emergence of K10, K16, and K17 and podoplanin. The study protocol for analyzing biopsy and cytology samples was reviewed and approved by the Ethical Board of Yamanashi Prefectural Central Hospital. Patients were not asked to give informed consent, because anonymous specimens and clinical data were used.

Categorization Using Bethesda System
The 2001 Bethesda System (TBS) for reporting cervical or vaginal cytology was the initial method used for cytological evaluations in a hospital setting. Among the 123 malignant cases, 107 (87.0%) were initially diagnosed as having malignant [9 with high-grade squamous intraepithelial lesion (HSIL) (7.3%), and 98 with SCC (79.7%)]]. Of the remaining 16 cases not correctly diagnosed as malignant, 5 (4.1%) were initially diagnosed as negative...
for intraepithelial lesions or malignancy (NILM), 1 (0.8%) with atypical squamous cells of undetermined significance (ASC-US), 3 (2.4%) with low-grade squamous intraepithelial lesion (LSIL), and 7 (5.7%) with ASC, though those do not exclude HSIL (ASC-H). These 16 malignant tumors occurred in the tongue, gingiva, buccal mucosa, or lip, as shown in Table I. Thus, the 1 expert cytologist and 3 pathologists who served as screeners reexamined those 16 cases by referring to the 53 non-neoplastic lesion cases, including 1 case of LSIL and 3 of ASC-H, all of which occurred in the gingiva as well as the 107 malignant cases above mentioned, to determine the cytological characteristics of oral SCC and CIS cells other than nuclear factors.

**Selection of Five Parameters for Cytologic Features**

While reviewing the 20 (16 malignant, 4 non-neoplastic) cases, each of which had samples available on 2–10 slides (Table I), the screeners listed the varieties of cytoplasmic appearance as possible cytological parameters by comparing histological features with special attention given to modes of keratinization. Furthermore, they again reviewed the remaining 107 malignant and 49 non-neoplastic cases in addition to these 20 cases for their cytological findings. As described in the Results section, we finally selected the following five parameters: (i) concentric arrangement of orangeophilic cells indicating keratin pearls; (ii) large number of orangeophilic cells; (iii) bizarre-shaped orangeophilic cells with a spindle- or caudate-shape with deep orange-yellow color and distinct cell borders, but without nuclear atypia; (iv) keratoglycoblules, a term used to designate small round cells with deep orangeophilic cytoplasm; and (v) uneven filamentous cytoplasm. We termed this cytologic evaluation method based on the presence or absence of these five parameters as the five parameter system (FPS).

**Reevaluation by TBS and FPS**

Among the 16 malignant cases (12 SCC, 4 CIS), which were selected for the reevaluation test, 2 with SCC and 3 with CIS were initially diagnosed as NILM, 1 with SCC as ASC-US, 3 with SCC as LSIL, and 6 with SCC and 1 with CIS as ASC-H, while the 4 non-neoplastic cases were diagnosed as LSIL or ASC-H (Table I). Each case had 2–10 slide specimens available (Table I), thus only 1 from each was selected by choosing a slide that either contained a reasonable amount of cells or the one with the larger numbers of cells when only 2 or 3 slides were available, for a total of 20 slides from the 20 cases examined. This selection of slides for the reevaluation test was performed by the first author and corresponding authors. To avoid bias in the selection, it was not reconfirmed whether all five of the parameters were present. Reevaluations were then performed in a blind fashion by 4
examiners, 2 experts with >20 years of experience each as a cytologist and 2 nonexperts with <2 years of experience each. One of the expert cytologists, the first author, was involved in the screening of the 20 cases and fixed the 5 parameters for oral SCC as described above. Since the other 3 examiners were not familiar with the 5 cytological parameters noted above, they underwent comprehensive training, during which slides containing the 5 parameter features from the other 107 cases, that had correctly been diagnosed as malignant by TBS, of the 123 malignancies were presented to them, until they were able to recognize the parameters. The 20 slides used for reevaluation were not used for training the 3 examiners, hence they examined those for the first time during the reevaluation. A repeatability test was performed by asking the examiners for their evaluations of 20 specimens by use of TBS or by FPS, in which the presence (+) or absence (−) of parameters (i) (ii), (iii), (iv), and (v) were determined. Their final diagnoses were then compared between the two systems.

Statistical Analysis
Cytological parameter data were statistically analyzed using Fisher’s exact test with Graph-Pad Instat (version 3.06 for Windows; GraphPad Software, San Diego, CA). P-values <0.05 were considered to be statistically significant.

Results
TBS Evaluation
Of the 123 malignant cases, 107 (87.0%) had been correctly diagnosed as malignant by TBS, including 1 (0.8%) HSIL (severe dysplasia), 4 (3.3%) HSIL (severe dysplasia/CIS), 1 (0.8%) HSIL (CIS), 3 (2.4%) HSIL (CIS/SCC), and 98 (79.7%) SCC cases. However, the remaining 16 (13.0%) had not been diagnosed as malignant by TBS and were selected as the main materials for the present study, as described in Materials and Methods.

Cytology specimens from the 16 target cases commonly contained keratinizing superficial layer type (ST) and keratinizing intermediate layer type (IMT) cells, and rarely non-keratinizing ST/IMT/parabasal layer type cells. Interestingly, malignancy in these 16 cases was clinically doubtful, though histopathological findings later confirmed them to be malignant. Among the 53 non-neoplastic cases, 1 (1.9%) was categorized as LSIL, 3 as ASC-H (5.7%) (Table I), and the remaining 49 (92.5%) as NILM. Histopathologically, they were confirmed to be mucositis, granulation tissue (fibroepithelial polyp or pyogenic granuloma), or fistulas. Thus, the 4 non-neoplastic cases with nuclear atypia and the 16 malignant cases were selected for a reevaluation study.

Extraction of Parameters Specific to Oral SCC
In our review of cytology specimens from the 123 malignant cases including the 16 malignant cases selected for reevaluation and 53 non-neoplastic cases, including 4 normal control samples, we gave attention to not only nuclear but also cytoplasmic findings. As a result, 5 findings were considered to be most conspicuous in the 16 malignant but not in the non-neoplastic cases. In addition, these 5 findings were confirmed to be present in some of the remaining 107 cases. For this portion of the study, three of the four examiners who took the reevaluation testing were not involved. Those five parameters are described in detail in the following sections.

Concentric arrangement of orangeophilic cells indicating keratin pearls. Small clusters of keratinized cells were often found arranged in a concentric manner, which might represent a minimal formation of keratin pearls within oral SCC foci in HE-stained biopsy specimens (Fig. 1A, arrows). In cytology specimens, clusters of orangeophilic keratinized cells arranged in a concentric manner were often isolated from aggregates of smeared cells (Fig. 1B). In higher magnification observations, concentric arrangements of orangeophilic cells in cytology findings varied in size and shape (Figs. 1C–E), although they were mostly identical to each histological counterpart (Figs. 1F–H). SCC cells surrounding the concentric core of keratinized cells loosing nuclei had polygonal (Fig. 1F) or flat (Fig. 1G) shapes. Among those with a flat shape, surrounding flat cells were occasionally arranged in a concentric manner as squamous eddies or classic keratin pearls (Fig. 1H). Thus, these concentric arrangements of orangeophilic cells were considered to indicate an initial stage of keratin pearl development. When keratinized cells began to form concentric structures, they tended to detach from each other (Figs. 1C, F, and G), though they were densely packed in larger concentric structures (Figs. 1E and H). It was difficult to distinguish any nuclear atypia in those orangeophilic cells that formed concentric structures. Such concentric arrangement of keratinized cells was observed in 9 (75%) of the 12 SCC cases, whereas that was not seen (0%) in the 4 CIS and 53 non-neoplastic cases.

Large numbers of orangeophilic cells. Most of the cytology specimen smears from malignant cases contained cells with a monotone appearance and orangeophilic cytoplasm containing nuclei (Fig. 2A). Those orangeophilic cell clusters contained <10% nonorangeophilic cells, among which there were no green-stained basal or parabasal cells. With higher magnification, those keratinized cells consistently showed a low nuclear/cytoplasm (N/C) ratio (Fig. 2B). Thus, it was difficult to determine the malignant or neoplastic characteristics of these orangeophilic cells, though there was an
extraordinarily large number, which was never seen in the non-neoplastic lesion samples. In the corresponding tissue specimens, keratinized SCC cells were thickly stacked on the surface and formed keratin pearls in the center of the SCC cell foci. These keratinized SCC cells were present in the smears used for cytology (Fig. 2C). Thus, in such oral SCC cases with extremely thick keratin pearls, high cell density and monotone orangeophilic
cells with nuclei should be regarded as malignant hallmarks, even though their nuclei were small. Such large numbers of orangeophilic cells were observed in 12 (100%) of the 12 SCC cases and 3 (75%) of the 4 CIS cases, whereas that was not seen (0%) in the 53 non-neoplastic lesions.

Bizarre-shaped orangeophilic cells without nuclear atypia. In addition to the non-atypical orangeophilic cells noted above, orangeophilic cells with bizarre shapes, from spindle to caudate, were also seen in cytology specimens from oral SCC cases (Fig. 3A). Among the cells of the spindle, those with extremely long and condensed (deeply stained with orange) cytoplasm were conspicuous. With higher magnification, those cells had elongated nuclei without distinctive atypia, while some lacked nuclei (Fig. 3B). In HE-stained biopsy specimens from the corresponding cases, keratinized SCC cells with long spindle shapes were singularly detached from the surface of the SCC foci (Fig. 3C). These spindle-shaped surface cells sometimes appeared as characteristic bizarre-shaped SCC cells in cytology smears (Figs. 3A and B). Bizarre-shaped orangeophilic cells were seen in 10 (83.3%) of the 12 SCC cases and 3 (75%) of the four CIS cases, whereas none (0%) were observed in the 53 non-neoplastic cases.

Keratoglobules (small round-shaped cells with deeply stained nuclei and cytoplasm). Small round-shaped and orangeophilic cells in the smears were individually detached from green- or pink-stained squamous cells (Fig. 4A). Their cytoplasm was more deeply stained with orange G than the bizarre-shaped orangeophilic cells mentioned above, and atrophic nuclei were also deeply stained with hematoxylin (Figs. 4C–H). We designated these characteristic keratinized cells as “keratoglobules.” There were some variations in the shapes of these keratoglobules within the range of round to ovoid, while their nuclei also showed variations from karyorrhexis (Figs. 4C and D) to pyknosis (Figs. 4E and F) and karyolysis (Figs. 4G and H). Their shapes and sizes resembled those of basal/parabasal cells, while their nuclei resembled those of superficial cells. Such inconsistencies in cellular differentiation between nuclei and cytoplasm were considered to be malignant hallmarks. Histologically, keratoglobules were located in the superficial zone of oral SCC nests (Fig. 4B) and isolated from surrounding SCC cells, which were in tight contact with each other (Fig. 4B). Their shapes were obviously different from the flat- or spindle-shaped keratinized cells that formed keratin pearls, though their staining characteristics resembled those of keratin pearls (Figs. 1–3). Keratoglobules were seen in 12

Fig. 2. Predominant population of orangeophilic keratinized cell in oral SCC (case 2). A, B: Papanicolaou stain; C: HE stain. A: ×100, B: ×400, C: ×100. Most of the cells in the smears had a monotone appearance with orangeophilic cytoplasm containing nuclei (A). Higher magnification revealed no cellular atypia in these keratinized cells with a monotone appearance and a lower nuclear/cytoplasm (N/C) ratio (B). In surgical specimens, keratinized SCC cells were thickly stacked to form keratin pearls in the center of SCC cell foci (C). Thus, this level of cell density among orangeophilic cells with nuclei should be regarded as indicating malignancy in oral SCC cases. [Color figure can be viewed at wileyonlinelibrary.com]
Uneven filamentous cytoplasm. From well-differentiated SCC foci, orangeophilic SCC cells containing distinct nuclei without atypia formed aggregates in the smears (Fig. 5A). Histologically, the SCC foci were mainly composed of densely packicle cell-like cells with a wide but uneven eosinophilic cytoplasm (Fig. 5B). With higher magnification, the orangeophilic cells were found to contain wavy filamentous structures, which were irregularly distributed with either a thick (Fig. 5C), granular and fine wavy distribution in the periphery (Fig. 5D), along with oval condensation around the nuclei (Figs. 5C–F). Higher magnification of histological specimens showed that oral SCC cells were densely packed and each had intercellular bridges, to which thick cytoskeletal filamentous bundles entered from the cytoplasmic side, as if painted with a brush, and were condensed around the nuclei (Fig. 5G), which was consistent with the cytological features (Figs. 5C–F). The nuclei of these SCC cells with irregularly thick filamentous cytoplasm were larger and appeared to be viable (Fig. 5), as compared with those of the cells with keratin pearls/keratoglobules (Figs. 1 and 4). Such uneven filamentous cytoplasm was recognized in 12 (100%) of the SCC and 4 (100%) of the CIS cases, while that was seen in none (0%) of the non-neoplastic lesions.

Reevaluation by TBS and FPS

According to the TBS findings, all of the 12 well-differentiated SCC cases with no obvious nuclear atypia were determined by the 2 nonexperts to be either NILM (33.3%, 58.3%, respectively) or ASC-US (66.7%, 41.7%), and by the 2 experts to be NILM (0%, 16.7%), ASC-US (0%, 16.7%), LSIL (58.3%, 33.3%) or ASC-H (41.7%, 33.3%). Four CIS cases were diagnosed as NILM (50%, 100%) or ASC-US (50%, 0%) by the nonexperts, and as NILM (0%, 75%), LILSIL (25%, 0%), or ASC-H (75%, 25%) by the experts. In contrast, the 4 non-neoplastic cases with nuclear atypia were judged as ASC-H (100%, 75%) or SCC (0%, 25%) by the nonexperts, and as ASC-US (0%, 25%), LSIL (25%, 0%), or ASC-H (75%, 75%) by the experts. The highest grade of ASC-H was given to both malignant and non-neoplastic cases, though more frequently in non-neoplastic cases. These results indicated that diagnosis of the 16 cases without obvious nuclear
atypia was extremely challenging when using TBS (Table II).

In contrast, when using FPS, most of the malignant cases with at least 1 of the 5 parameters confirmed were not overlooked by any of the 4 examiner cytologists. As shown in Table II, detection rates were quite similar between the second expert and 2 nonexperts. All of the parameters were noted at higher rates in SCC (60–96%) and CIS (63–81%), except for parameter (i), which was not always present in malignant cases but never detected in reactive lesions by the 4 cytologists (Table II, rightmost column). The total numbers of the 5 parameters confirmed in each case (Table II, bottom of each lesion category) indicated that all parameters were not necessarily included in each malignant specimen. However, there were no differences in malignant judgment between the 2 experts and 1 of the nonexperts (12/12 SCC cases; 4/4 CIS), and nearly no differences with the other nonexpert (11/12 SCC; 4/4 CIS), although the nonexperts tended to identify fewer of the 5 parameters. In spite of those reduced numbers, the nonexperts gave a malignant judgment even when only a single parameter was noted, indicating that any of the 5 parameters is a helpful indicator of malignancy. In the CIS cases, at most 3 or 4 parameters were included, and the nonexperts tended to overlook them more frequently (3/4 cases). Nevertheless, as shown in Table II, none of the 5 parameters were identified in non-neoplastic cases, even those with nuclear atypism.

Discussion

The goal of this study was to determine cytological criteria specific to well-differentiated oral SCC and CIS, which lack discernible nuclear atypia, because their characteristic of no or few atypical features tends to lead to false-negative diagnoses.18 To this end, we newly determined 5 parameters for cytoplasmic features of well-differentiated (keratinized) oral SCC cells after comparative examinations of cytological and histological findings. The present trial was successful for discriminating SCC cells in cytology specimens based on their cytoplasmic characteristics. An advantage of FPS is that it can widen the value of cytological examinations for oral SCC cases, most of which are well differentiated and difficult to precisely diagnose based only on nuclear features shown by TBS.

This study showed that TBS functioned well for oral cases (119 SCC, 4 CIS) that had nuclear atypia, while it did not function well for 16 (13%) cases with cytology specimens showing no obvious nuclear atypia. Similar results were previously reported by Koch et al.,26 who were able to cytologically detect 92 (88.5%) of 104 malignant (SCC and CIS) cases that were histologically

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**Fig. 4.** Keratoglobules in oral SCC (case 10). A, C–H: Papanicolaou stain; B: HE stain. A: ×400, B: ×200, C–H: ×400. Singular small round-shaped and orangeophilic cells were found detached from green- or pink-stained squamous cells in the smears (A). The cytoplasm of these cells was deeply stained with orange G, while atrophic nuclei were also deeply stained with hematoxylin (C–H). These characteristic keratinized cells were designated in this study as “keratoglobules.” They showed some variations in shape from round to ovoid, while their nuclei also showed variations from karyorrhexis (C, D) to pyknosis (E, F), and karyolysis (G, H). Histologically, keratoglobules were located in the superficial zone of oral SCC nests and isolated from surrounding SCC cells, which were in tight contact with each other (B). These were obviously different from flat or spindle-shaped keratinized cells that formed keratin pearls (shown in Figs. 1–3). [Color figure can be viewed at wileyonlinelibrary.com]
confirmed. Even after a second review, they did not find any evidence of malignancy in 8 of the remaining 12 cases. Thus, it is possible to conclude that at least 10% of oral SCC/CIS cases may be overlooked in cytological evaluations that depend on TBS findings, because >10% of oral SCC/CIS cells lack nuclear atypia.

TBS was mainly devised for gynecological cytology, in which ASC-US and LSIL are not objects for a biopsy or close examination. As confirmed in the present re-evaluation test results, cytologists not trained for oral cancer have difficulty at times detecting well-differentiated SCC/CIS cells. In cases of cervical cancer, HPV tests are performed when a diagnosis of ASC-US or LSIL is made, while HPV-positive (+) cases are further examined using biopsy findings and HPV-negative (−) cases are cytologically reexamined after 1 year. However, oral surgeons must maintain suspicion regarding oral cancer even after repeated cytology examinations for cases with clinically suspected malignancy when they receive a diagnosis of NILM, ASC-US, or LSIL, because HPV test results are not informative for oral malignancy cases, as they are not always infected by HPV. Thus, it is important for oral surgeons and cytologists/pathologists to communicate regarding patients with clinically suspected malignancy, otherwise those would not be subjected to follow-up examinations until lesions developed in a later stage. This is because HPV-positive head and neck SCCs are thought to be classified as nonkeratinized or less-differentiated histological types, and arise in the oropharynx. We consider that obtaining cytology results from well-differentiated SCC is challenging, thus HPV tests are not always required in cases of oral SCC, as most of those

Fig. 5. Uneven filamentous cytoplasm of orangeophilic cells in oral SCC (case 9). A, C–F: Papanicolaou stain; B, G: HE stain. A: ×100, B: ×4, C–G: ×400. Some orangeophilic SCC cells were characterized by a filamentous cytoplasm appearance in both (A) cytology and (B, G) histology findings. Cytoskeletal filaments were unevenly distributed in the cytoplasm, with a granular or fine wavy appearance in the periphery, and condensed around the nucleus (C–F). In the most extreme instances, oral SCC cells with a prickle-cell-like appearance were compactly packed and connected with intercellular bridges, with thick cytoskeletal filamentous bundles entering from the cytoplasm, as if painted with a brush (G). Those thick filaments were more condensed in perinuclear space, which was consistent with the cytological features (C–F). [Color figure can be viewed at wileyonlinelibrary.com]
are well differentiated. Indeed, the 20 samples we investigated in this study were obtained from the tongue, gingiva, buccal mucosa, and lip, while none came from the oropharynx (Table I).

Previous diagnostic cytology methods have not focused on keratin pearls even in examinations of the oral cavity. Among the 5 cytological features proposed for FPS, the characteristic concentric arrangement of orangeophilic cells (parameter i) indicating keratin pearls was observed in 50% of the SCC cases, while that was not found in any CIS or reactive lesion cases. This is because cancer pearls are located in the deeper portion of CIS lesions, and may not be easily included in cytology specimens. Recently, Watanabe et al. also reported that

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<th>Lesions</th>
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<th>Diagnostic categories (TBS)/ criteria used for diagnoses (FPS)</th>
<th>Diagnosed case numbers by reviewers</th>
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<td>Squamous cell carcinoma (SCC): 12 cases</td>
<td>Bethesda (TBS)</td>
<td>NILM 0 2 4 7 13 (27) ASC-US 0 2 8 5 15 (31) LSI 7 4 0 11 (23) ASC-H 5 4 0 9 (19) HSIL 0 0 0 0 (0) SCC 0 0 0 0 (0)</td>
<td>Expert 1 (first author) 0 0 0 0 0 (0) Expert 2 1 0 1 0 2 (12) Nonexpert 1 2 1 1 1 3 (6) Nonexpert 2 3 2 3 4 11 (23) Total 12 12 11 11 46 (96)</td>
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<td>Five Parameter (FPS) i 9 7 7 6 29 (60) ii 12 10 8 12 42 (88) iii 10 8 9 10 37 (77) iv 12 11 8 9 40 (83) v 0 0 0 0 0 (0)</td>
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<td>Total number of five parameters recognized in each case 0 0 0 0 1 (2) 1 0 1 0 1 (2) 2 0 1 1 1 (3) 3 2 2 3 4 (11) 4 1 8 3 7 (19) 5 0 0 0 0 (0)</td>
<td>12 12 11 11 46 (96)</td>
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<td>NILM 0 3 2 4 9 (56) ASC-US 0 0 2 0 2 (13) LSI 3 1 0 0 4 (25) ASC-H 0 0 0 0 (0) HSIL 0 0 0 0 (0) SCC 0 0 0 0 (0)</td>
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<td>Cases diagnosed as NILM 4 4 3 3 14 (88)</td>
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<td>Cases diagnosed as NILM 4 4 4 4 16 (100)</td>
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Table II. Comparative Evaluation of 16 Malignant and 4 Reactive Lesions of the Oral Mucosa between TBS and FPS
they failed to find keratin pearls in oral CIS specimens. In endocervical (EC) smears, epithelial cells without nuclear atypia contained in keratin pearls have been simply reported as “parakeratotic cells,” the same as those in non-neoplastic lesions, without being distinguished from each other. This fact clearly indicates that differential diagnosis between malignant and benign EC cases is dependent on nuclear atypia. However, such a concentric arrangement of orangeophilic cells does exist in EC smears, as shown in Fig. 1.39 in the chapter by Abdultric et al. and in Fig. 2.15a (squamous pearl, example of typical parakeratosis) in the Bethesda 3rd edition, though that has been routinely ignored or judged as ASC-US. As revealed in this study, detection of keratin pearls is a reliable method for definitive diagnosis of oral SCC.

The second feature of FPS is a predominant population of keratinized cells (parameter ii). Nuclear atypia should be minimized in oral SCC cells during the keratinization process, with keratinized cells without nuclear atypia eventually becoming predominant. Such a tendency towards keratinization is a very strong characteristic of oral SCC. Bizarre-shaped orangeophilic cells without nuclear atypia (parameter iii) were also found to be characteristic of oral SCC and CIS, while they were never found in reactive lesion samples. In EC smears, these orangeophilic cells may only be recognized as keratotic changes (parakeratosis or atypical parakeratosis). According to the 3rd edition of TBS 2015, they must be diagnosed in a range that includes NILM, ASC-US, and LSIL, but not as HSIL. However, when nuclear atypia is evident, they are regarded as keratinizing SCC. Meanwhile, in oral cytology findings, such bizarre-shaped orangeophilic cells without nuclear atypia have not been documented. We consider that these cell types can be a very specific criterion of oral malignancy and likely originate from stacks of keratinized SCC foci on the surface.

Keratoglobules (parameter iv), isolated and small round-shaped keratinized cells containing pyknotic nuclei, were observed in all of the present SCC and CIS cases, but never in reactive lesion samples. In addition to pyknosis, nuclear shapes were interpreted as karyolysis or karyorrhexis. Any type of nuclear morphology may indicate the process of cell death in association with keratinization, though it remains unknown how nuclei are cleared in those cells. Nevertheless, these nuclear shape varieties are definitely different from apoptosis or any other already-known cell death machinery.

Due to hemorrhage resulting from collapsed intraepithelial blood vessels in oral CIS and SCC cases, because hemaphagocytosis induces keratinization of epithelial cells facing connective tissues. In gynecological cytology, keratinizing basaloid cells are often seen in non-neoplastic lesions that originate from senile atrophy, and miniature squamous cells with small bland and pyknotic nuclei are considered to represent typical parakeratosis (Fig. 2.15b in the Bethesda 3rd edition).

Uneven filamentous cytoplasm (parameter v) was identified in 100% of the 16 malignant cases, whereas that was not seen in any of the reactive lesions. This cytological feature may represent dyskeratotic changes among IMT cells in the lower prickle cell layers or singular cellular keratinization among those in the basal half of CIS/SCC foci, in which keratin filaments (tonofilaments) are not evenly distributed. Such dyskeratotic cells were found to contain definite and large nuclei with clear nucleoplasm and intercellular bridges in the present study, which is in sharp contrast to keratoglobules with characteristic nuclear collapse shapes of pyknosis, karyolysis, or karyorrhexis. Both keratoglobules and uneven filamentous cytoplasm were simultaneously identified in 100% of the 16 SCC and CIS cases, while keratin pearls were found in 60 and 0%, respectively. Uneven filamentous cytoplasm is considered to be an initial sign of abnormal keratinization among activated keratinocytes, which may secondarily accelerate keratinization with nuclear collapse.

In conclusion, FPS is a powerful tool for cytological diagnosis of well-differentiated oral SCC. When some of the five parameters are detected, even though all may not be simultaneously identified, it is possible to make a diagnosis of malignancy. We consider that FPS is much more effective than TBS for diagnosis of oral SCC and CIS specimens lacking nuclear atypia. However, the present series of specimens submitted to reexamination included only 20 cases, thus it will be necessary for us to perform further studies to test the diagnostic value of FPS. In addition, we also anticipate that FPS will be widely investigated by other groups to confirm its usefulness for oral SCC cytology examinations.

References


