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ORIGINAL ARTICLE

Contribution of transglutaminases and their substrate proteins to the formation of cornified cell envelope in oral mucosal epithelium

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Cornified envelope formation is crucial for the final differentiation of keratinized epithelium. However, the mechanisms of cornified envelope formation in the oral epithelium remain unclear. The aim of this study was to clarify the differences in the distribution and expression of cornified envelope related proteins and genes between keratinized and non-keratinized oral epithelia. We immunohistochemically investigated the distribution patterns of transglutaminase 1 (TG1), transglutaminase 3 (TG3), and their substrate proteins involucrin (IVL), loricrin (LOR), and small proline rich proteins (SPRs), in 19 keratinized and 14 non-keratinized oral epithelium samples. TG1 and TG3 mRNA levels were investigated in both types of epithelium by real time reverse transcription polymerase chain reaction (RT-PCR) using paraffin-embedded specimens. Data were analyzed to identify factors involved in cornified envelope formation. We demonstrate that 11 localization patterns show statistically significant differences between keratinized and non-keratinized oral epithelia. These factors clearly drove the separation of the two groups during cluster analysis. TG1 mRNA levels in keratinized oral epithelium were significantly higher than those in non-keratinized oral epithelium. In conclusion, the characteristic distribution of transglutaminases and their substrates and the mRNA levels of TG1 can regulate cornified envelope formation in keratinized oral epithelium, together with the contribution of TG3 first reported in this paper.

KEYWORDS
cornified envelope, keratinized oral epithelium, non-keratinized oral epithelium, substrates, transglutaminase

INTRODUCTION

During terminal differentiation of stratified squamous epithelium in the epidermis and of keratinized oral epithelium, an insoluble structure of cross-linked proteins, called the cornified envelope, is formed just beneath the plasma membrane (1–3). Component proteins are cross-linked by the transglutaminase enzyme to form the cornified envelope (4,5). Transglutaminases are a calcium-dependent enzyme family (2) that catalyzes the formation of isopeptide and ester bonds between glutamine and lysine residues in various types of substrate proteins (6). Transglutaminase 1, 3, and 5 (TG1, TG3, and TG5) are thought to participate in cross-linking during formation of the cornified envelope in the epidermis (7–10). Several proteins, including involucrin (IVL), loricrin (LOR), and small proline rich proteins (SPRs), have been implicated as cornified envelope precursor proteins (3,11–13). The expression of TG1 and TG3 is regulated by TGM1 and TGM3, which are located on chromosome 14q.11.2 (14) and chromosome 20q11.2 (15), respectively. These are the main

players in the assembly of the cornified envelope and in the maintenance of epidermal structure and function, and they are distributed in the upper spinous and granular layers of the epidermis along with precursor proteins (9,10,16,17). Initial cornified envelope formation is mediated by TG1-dependent cross-linking of IVL (6) and subsequent TG3-dependent cross-linking of LOR and SPRs during terminal differentiation of the epidermis (18).

In normal epidermis, IVL, a common component of the cornified envelope (11), is distributed in the cytoplasm of the granular and upper spinous layers (10,19). SPRs, a family of 12 related proteins, form bridges between LOR molecules (13). SPR1 and SPR2 are present in the cytoplasm of the upper spinous and granular layers, whereas SPR3 is absent (12,20,21). LOR is a poorly soluble protein found in the granules of the granular layer in the epidermis and it is released from the granules and cross-linked to the inner surface of developing cornified envelope during the terminal phase of differentiation. In the epidermis, LOR is the major component of cornified envelope formation (18,22).

As for oral transglutaminases and their substrates, several reports showed cytoplasmic distribution of IVL in the upper third of the epithelium in the palatal and gingival mucosa (23) and in all suprabasal layers in the buccal mucosa (20,24,25), as well as showing cytoplasmic distribution of SPR3 in the spinous layer in the buccal (20,21), lingual, and palatal mucosa (21). Moreover, one animal experiment showed the distribution of SPR1 and SPR3 in the tongue and lip mucosa (26). Lee *et al*. (27) investigated the expression of cornified envelope proteins from human gingiva and buccal mucosa and proposed that SPR1a/b constituted 60%–70% of cornified envelope proteins, together with a small amount of IVL and other cornified envelope proteins. Lee *et al*. also reported a lower TG3 and LOR content, but a higher SPR content in gingival cornified envelope in comparison to those in the epidermal cornified envelope (27). However, studies on the distribution of transglutaminases and their substrates in the oral mucosa are limited. Some investigators reported membranous distribution patterns of TG1 in the hard palate (16) and buccal mucosa (16,25) and cytoplasmic and nuclear distribution of TG3 form the lower spinous layer to the superficial layer in the buccal mucosa (25) and in the esophageal mucosa (28). However, we have not fully understood the distribution of those proteins in either keratinized or non-keratinized oral epithelia.

Their abnormalities, such as the mutation of TG1 and autoantibody production against TG3, cause ichthyosis (29) and dermatitis herpetiformis (30), respectively. In oral regions, some reports have shown that abnormal distribution and mRNA levels of TG1 and TG3 might be related to periodontitis (31), oral premalignant lesion, and oral squamous cell carcinoma (32–34). To clarify the mechanisms of oral diseases, including abnormal keratinization and oral squamous cell carcinomas, it is necessary to know how TGs and their substrates contribute to the differentiation and keratinization of normal human oral epithelium. However, the contributing factors and mechanisms of cornified envelope formation in normal oral mucosal epithelium remain unclear, especially in humans. Therefore, the aim of the present study was to clarify the differences in the distribution and expression of cornified envelope related proteins and genes between keratinized and non-keratinized oral epithelia. We investigated the distribution patterns of TG1 and TG3 and their substrates (IVL, LOR, and SPRs) in keratinized and non-keratinized oral epithelia using immunohistochemical and molecular approaches.

MATERIAL AND METHODS

Sample selection

We collected formalin fixed (24–48 h) paraffin embedded (FFPE) oral mucosal samples, including gingival, hard palate, cheek, and lateral tongue samples, archived in the surgical pathology laboratory of the Matsumoto Dental University Hospital. We then examined hematoxylin and eosin (HE) stained sections for their keratinization pattern, rete ridge structure, polarization of basal cells, cellular atypia, and abnormal mitotic figures. Samples that were considered normal in HE stained sections (Figure 1A–D) were then stained for the cell proliferative marker Ki-67 and the basal cell marker of normal non-keratinized oral epithelium cytokeratin 19. Finally, we selected 19 keratinized and 14 non-keratinized oral epithelium samples that were phenotypically within the normal range (Figure S1). The age of the sources of our samples ranged from 35 to 75 years, with an average age of 59 years. We used normal epidermis as a positive control for immunohistochemical analysis. Our study was approved by the Ethics Committee of Matsumoto Dental University (approval number 209) and conducted according to the principles of the Declaration of Helsinki (version 2008). As all samples were archived as paraffin-embedded tissues, informed consent was obtained in the form of opt-out.

Immunohistochemistry

Three-micrometer thick sections were made from FFPE tissue blocks, and subsequently deparaffinized and hydrated. We determined an optimal antibody dilution and an optimal antigen retrieval method for each antibody using appropriate positive control specimens (that is, the normal epidermis). Sections for IVL and LOR detection, but not those for TG1, TG3, SPR1b, and SPR3 detection, were treated with proteinase K, while sections for SPR1a detection were heat treated

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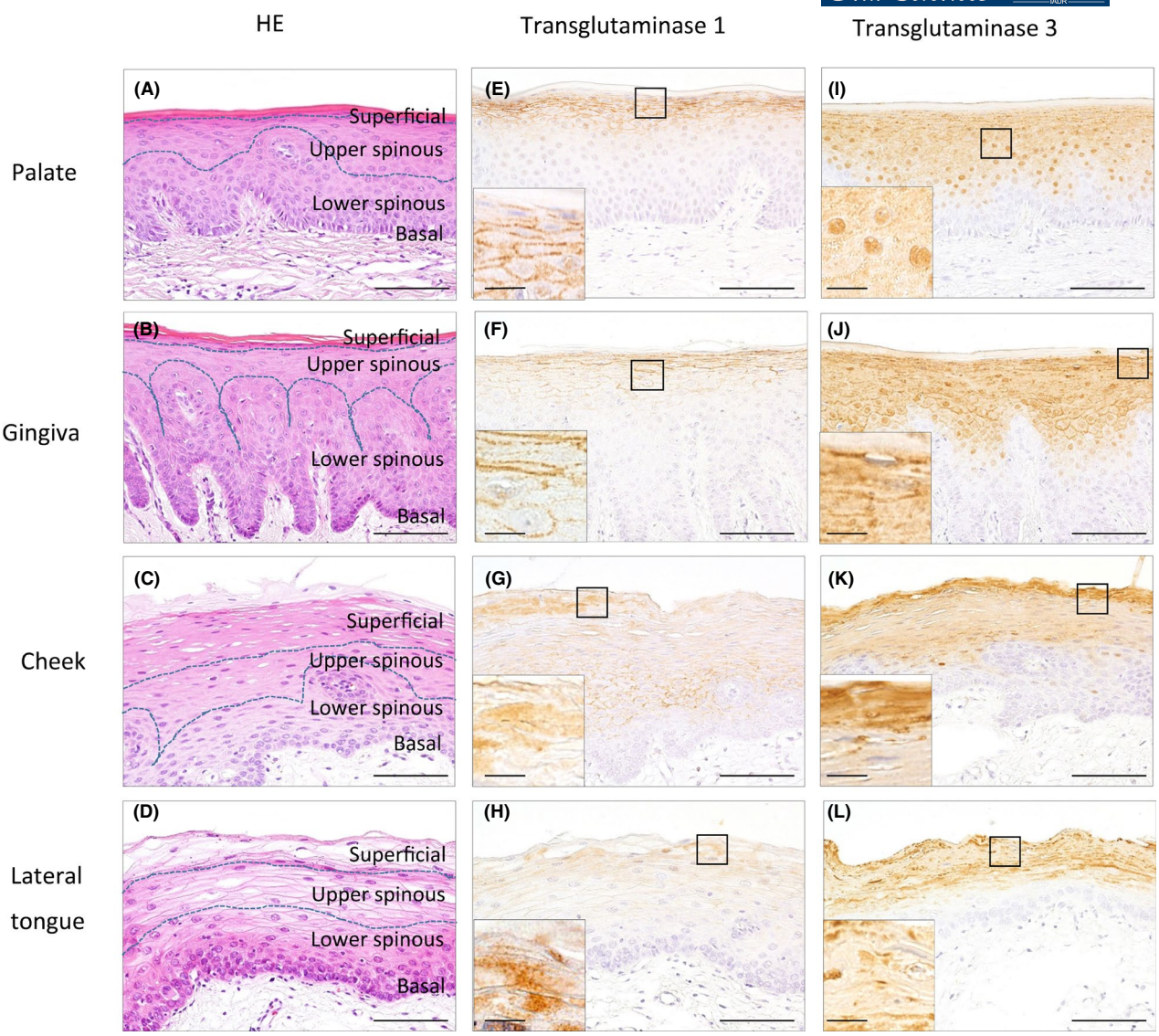

FIGURE 1 Microphotographs of HE, TG1, and TG3 immunostains. HE-stained sections of superficial, upper spinous, lower spinous, and basal layers (A–D). TG1 (E–H) and TG3 (I–L) localization in the hard palate (A, E, I), gingiva (B, F, J), cheek (C, G, K), and lateral tongue (D, H, L). Scale bars represent 100 and 20 μm in the main panels and insets, respectively. The small square boxes of the main panels indicate the site of insets.

in an autoclave with 0.01 M sodium citrate buffer (pH 6.0). After blocking endogenous peroxidases with a 3% H_2O_2 solution and nonspecific reactions of Ig by protein block (DAKO, Glostrup, Denmark) for 15 min, all sections were incubated with primary antibodies. The primary antibodies with their dilutions and incubation times were as follows: TG1, 1:200, 1 h at room temperature (Rabbit polyclonal antibody, Proteintech, Rosemont, IL, USA, Code: 12912-3-AP); TG3, 1:10,000, overnight at 4°C (Mouse monoclonal antibody, clone C2D; Covalab, Villeurbanne, France, Code: mab0057-P); SPR1a, 1:250, overnight at 4°C (Rabbit polyclonal antibody; Biorbyt, Cambridge, UK, Code: orb1053); SPR1b, 1:100, 1 h at room temperature (Rabbit polyclonal antibody; Abgent, San Diego, CA, USA, Code: AP9052b); SPR3, 1:250, 1 h at room temperature (Mouse monoclonal antibody, clone 4A12, Abnova, Taipei, Taiwan, Code: H00006707-M01); IVL 1:200, 30 min at room temperature (Mouse monoclonal antibody, clone SY5; ScyTek Laboratories, Piscataway, NJ, USA, Code: RA0166-C.5); LOR, 1:1000, 1 h at room temperature (Rabbit polyclonal antibody; GeneTex, Irvine, CA, USA, Code: GTX116013). After washing three times with phosphate buffered saline (PBS), all slides were incubated with secondary antibody for 30 min. Nichirei MAX-PO Multi (host-Goat; Nichirei, Tokyo, Japan) was used as secondary antibody. After visualization with 3-3´-diaminobenzidine tetra hydrochloride (DAKO), sections were counterstained with hematoxylin. For the preparation of negative control slides, we omitted the primary antibody and followed the other procedures as mentioned earlier, to check for nonspecific reactions.

Cytoplasmic and membranous reactions of all antibodies were evaluated in four layers of the epithelium: the basal, lower spinous, upper spinous, and superficial layers. If the primary antibody positively reacted with ≥50% or <50% of cells of each layer, we considered the sample positive or negative, respectively. Nominal values were analyzed by descriptive statistics using a 95% confidence interval.

Real-time polymerase chain reaction

We extracted RNA from all our samples using the RNeasy FFPE kit (Qiagen, Hilden, Germany). cDNA was synthesized with SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), TG1 and TG3 mRNA levels were analyzed using an Eco Real-Time PCR system (Illumina, San Diego, CA, USA) using a TB Green Premix Ex Taq II (Tli RNaseH Plus) kit (Takara Bio, Shiga, Japan). The sequences of primer sets were as follows: for TG1, the forward primer was 5´-CCCCCGCAATGAGATCTACA-3´ and the reverse was 5´-ATCCTCATGGTCCACGTACACA-3´; for TG3, the forward primer was 5´-GACAAGTTCTCCAGCCAGGAG-3´ and the reverse primer was 5´-AGTGGAAACACAGCCTT CGTC-3´; for *β-actin*, the forward primer was 5´-CCTGGCACCCAGCACAAT-3´ and the reverse primer was 5´GGGCCGGACTCGTCATACT-3´. Real-time PCR was performed in a final volume of 10 µl, in which 2.5 ng of cDNA was amplified in 5 µl of TB Green Premix Ex Taq with 10 µM of each primer for the target gene. We used *β-actin* as a reference gene. We did not aim to choose another common housekeeping gene, such as *GAPDH* and the 28S rRNA gene during the calculation of the $2^{-\Delta\Delta Ct}$. The first reason is that the expression of the *GAPDH* gene fluctuates across the stages of squamous epithelial differentiation (35–37); the second reason is that our samples showed 28S rRNA degradation, as indicated by the "RNA Integrity Number" calculated by an Agilent 2100 Bioanalyzer (Agilent, Tokyo, Japan). Thermal cycling conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 30 s and 60°C for 30 s, and by dissociation at 95°C for 15 s.

Statistical analysis

To compare keratinized and non-keratinized oral epithelium samples, localization and distribution values were analyzed using univariate and hierarchical cluster analyses. The relative gene expression levels were determined as $2^{-\Delta\Delta Ct}$ values in order to detect the fold change of the expression of target genes in keratinized and non-keratinized oral epithelium samples with respect to a normal lateral tongue sample. Relative gene expression values were analyzed using two-sample *t*-tests, Pearson's correlation tests and hierarchical cluster analysis. All tests were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (www.r-project.org) (The R Foundation for Statistical Computing, Vienna, Austria) (38). *p*-values <0.05 were considered significant.

RESULTS

Localization patterns of cornified envelope related proteins

TG1

In keratinized oral epithelium, positive reactivity to TG1 was found on cell membranes (100% of cases, 95% CI = 0.854–1) of the upper spinous cell layer but seldom in the cytoplasm (26% of cases, 95% CI = 0.091–0.512). The superficial layer was devoid of any visible staining (Figure 1E,F). Conversely, in non-keratinized oral epithelium, TG1 was variably detected on cell membranes (66% of cases, 95% CI = 0.289–0.823) and in the cytoplasm (42% of cases, 95% CI = 0.128–0.649) of the upper spinous layer, as well as in the cytoplasm (58% of cases, 95% CI = 0.23–0.77) and on membranes (16% of cases, 95% CI = 0.018–0.428) of the superficial layer. Consequently, 23% of cases (95% CI = 0.047–0.508) showed complete negativity in all layers (Figure 1G, H).

TG3

In keratinized oral epithelium, TG3 positivity was observed in the cytoplasm (100% of cases, 95% CI = 0.854–1) and on membranes (66% of cases, 95% CI = 0.384–0.837) of the upper spinous layer, as well as in the cytoplasm of the surface layer (31% of cases, 95% CI = 0.126–0.566) (Figure 1I,J). In contrast, in non-keratinized oral epithelium, only cytoplasmic reactivity to TG3 was detected in the upper spinous layer (100% of cases, 95% CI = 0.807–1) and in the superficial layer (92% of cases, 95% CI = 0.661–0.998), but no membranous reactivity was observed (Figure 1K,L).

IVL

In keratinized oral epithelium, IVL was localized on cell membranes (74% of cases, 95% CI = 0.488–0.909 and 68% of cases, 95% CI = 0.434–0.874) and in the cytoplasm (100% of cases, 95% CI = 0.854–1 and 100% of cases, 95% CI = 0.854–1) of the lower and upper spinous layers, without any reaction in the superficial layer (Figure 2A,B). In

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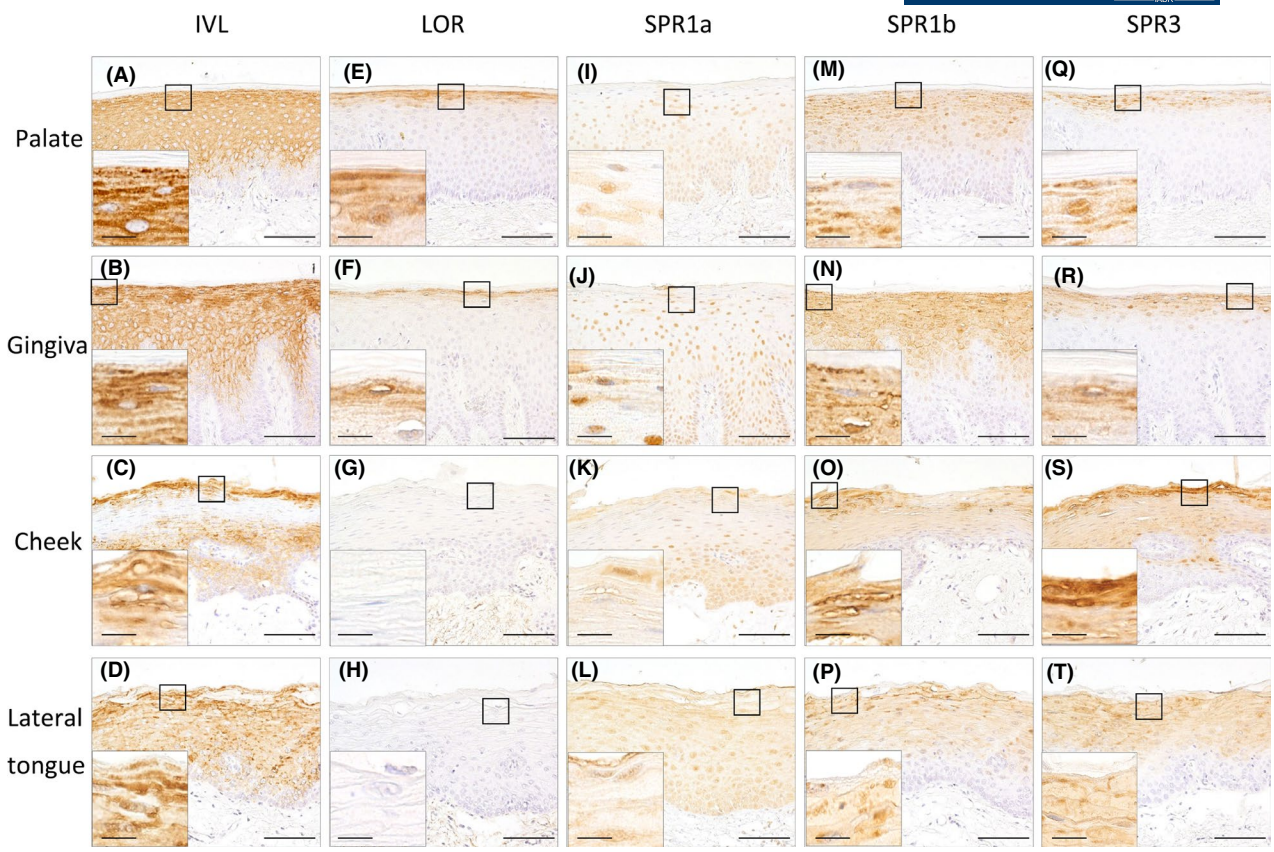

FIGURE 2 IVL, LOR, SPR1, SPR1a, SPR1b, and SPR3 immunostains. Localization of IVL (A–D), LOR (E–H), SPR1a (I–L), SPR1b (M–P), and SPR3 (Q–T) in hard palate (A, E, I, M, Q), gingiva (B, F, J, N, R), cheek (C, G, K, O, S), and lateral tongue (D, H, L, P, T). Scale bars represent 100 and 20 μm in the main panels and insets, respectively. The small square boxes of the main panels indicate the site of insets.

non-keratinized oral epithelium, cytoplasmic reactivity on the lower and upper spinous layers (100% of cases, 95% CI = 0.807–1 and 92% of cases, 95% CI = 0.661–0.998) and superficial layers (92% of cases, 95% CI = 0.661–0.998) were observed. IVL-positive membranes were not noted, except for only one case in the upper spinous layer (7% of cases, 95% CI = 0.002–0.339) (Figure 2C,D).

LOR

Only the upper spinous layer (84% of cases, 95% CI = 0.604–0.966) in keratinized oral epithelium showed cytoplasmic LOR localization (Figure 2E,F). In contrast, non-keratinized oral epithelium was devoid of any positive reaction (Figure 2G,H).

SPR1a

Both keratinized oral epithelium and non-keratinized oral epithelium showed very mild or faint positivity for SPR1a in nuclei and cytoplasm from the suprabasal layer to the superficial layer (Figure 2I,L). Moreover, SPR1a did not show any significant difference in distribution between keratinized and non-keratinized oral epithelia.

SPR1b

In keratinized oral epithelium, cytoplasmic (94% of cases, 95% CI = 0.74–0.999) and membranous (26% of cases, 95% CI = 0.128–0.649) reactivity to SPR1b was observed in the upper spinous layer, but not in the lower spinous layer. The superficial layer (95% of cases, 95% CI = 0.74–0.999) was devoid of any positive reaction (Figure 2M,N). In contrast, cytoplasmic reaction for SPR1b was found in the upper (92% of cases, 95% CI = 0.661–0.998) and lower (35% of cases, 95% CI = 0.128–0.649) spinous layers without superficial membranous reaction (100% of cases, 95% CI = 0.807–1) in non-keratinized oral epithelium (Figure 2O,P).

SPR3

In keratinized oral epithelium, SPR3 positive reaction was noted in the cytoplasm (100% of cases, 95% CI = 0.854–1)

of the upper spinous layer, but rarely on membranes (5% of cases, 95% CI = 0.001–0.26). The superficial layer (100% of cases, 95% CI = 0.854–1) did not show any positive reaction (Figure 2Q,R). In contrast, in non-keratinized oral epithelium, cytoplasmic reactions were observed in the superficial (85% of cases, 95% CI = 0.572–0.982), upper spinous (100% of cases, 95% CI = 0.807–1), and lower spinous (35% of cases, 95% CI = 0.128–0.649) layers (Figure 2S,T).

Statistical analysis of cornified envelope related protein localization

Data on the localization of TGs and their substrates in keratinized oral epithelium and non-keratinized oral epithelium were analyzed. Univariate analysis revealed that the distribution patterns of TG1, TG3, IVL, LOR, SPR1b, and SPR3 showed some statistically significant differences between keratinized and non-keratinized oral epithelia, while those of SPR1a did not (Table 1).

Although it was not possible to separate keratinized and non-keratinized oral epithelium samples by cluster analysis when only TGs were considered (data not shown), cluster analysis based on the localization of substrates (Figure S2A) and of TGs with all substrates (Figure S2B) led to the clear separation of all samples into two groups consisting almost exclusively of keratinized and non-keratinized oral epithelium samples. Each group contained only one exceptional sample. On the other hand, 11 factors listed in Table 1 were clearly divided all samples into two groups without any exception (data not shown). Furthermore, we checked the effects of various combinations of the factors listed in Table 1 on the output of cluster analysis. As a result, the top six or more factors with the smallest *p*-values in differential distribution analysis could drive the complete separation of all samples into keratinized oral epithelium and non-keratinized oral epithelium groups (Figure 3).

Statistical analysis of TG gene expressions

TG1 and TG3 mRNA was successfully amplified in 26 and 19 samples, respectively, out of 32 samples of either keratinized or non-keratinized oral epithelia. In the keratinized group, 12 samples failed to produce TG3 amplicons, and six of them also failed to produce TG1 amplicons. Log-transformed

TABLE 1 Univariate analysis based on localization of TGs and substrates in keratinized vs. non-keratinized group.

Variable	Reaction status	Keratinized	Non-keratinized	*p* values
IVL superficial cytoplasm	Positive case	0	13	<0.001
	Negative case	19	1	
SPR3 superficial cytoplasm	Positive case	0	12	<0.001
	Negative case	19	2	
LOR upper spinous cytoplasm	Positive case	16	0	<0.001
	Negative case	3	14	
IVL lower spinous membrane	Positive case	14	0	<0.001
	Negative case	5	14	
TG3 upper spinous membrane	Positive case	12	0	<0.001
	Negative case	7	14	
TG1 superficial cytoplasm	Positive case	0	7	<0.001
	Negative case	19	6	
IVL upper spinous membrane	Positive case	13	1	<0.001
	Negative case	6	13	
TG3 superficial cytoplasm	Positive case	6	13	<0.001
	Negative case	13	1	
TG1 upper spinous membrane	Positive case	19	8	0.006
	Negative case	0	5	
SPR1b lower spinous cytoplasm	Positive case	0	5	0.008
	Negative case	19	9	
SPR3 lower spinous cytoplasm	Positive case	0	5	0.008
	Negative case	19	9	

Abbreviations: IVL, Involucrin; LOR, Loricrin; SPR1b, Small proline rich protein 1b; SPR3, Small proline rich protein 3; TG1, Transglutaminase 1; TG3, Transglutaminase 3.

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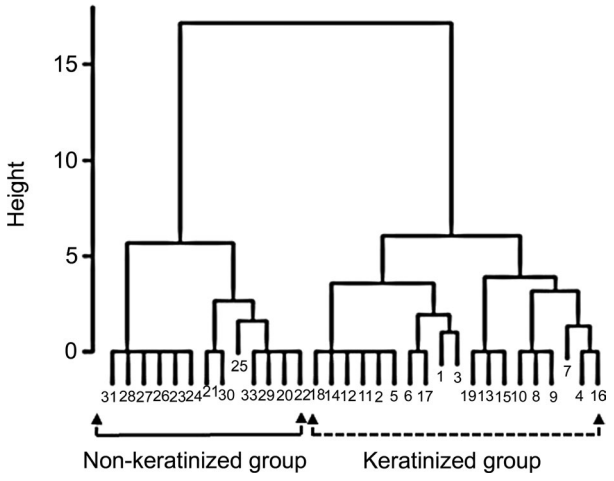

FIGURE 3 Cluster analysis of the distribution of transglutaminases and their substrates. Cluster dendrogram based on the top six factors with smallest *p*-values in univariate analysis (Table 1) showing complete separation of keratinized oral epithelium and non-keratinized oral epithelium groups. The non-keratinized group and the keratinized group are indicated by solid and dotted brackets, respectively.

parametric data of relative expression of TG1 and TG3 are shown as box plot graphs in Figure 4 A1 and A2, respectively, in the two groups of epithelia. Statistically, TG1 expression was significantly higher in keratinized oral epithelium than in non-keratinized oral epithelium (mean value 1.73 ± 0.74 vs. 0.77 ± 0.54, $p < 0.005$). TG3 also showed a tendency towards higher expression in keratinized oral epithelium than in non-keratinized oral epithelium (mean value 1.06 ± 0.62 vs. 1.01 ± 0.43, $p = 0.83$).

Cluster analysis based on TG1 mRNA levels roughly separated samples into two groups that were composed of 6 non-keratinized oral epithelium samples and 11 keratinized oral epithelium samples out of 8 and 17 samples, respectively (Figure 4B). Namely, both groups contained two and six exceptional samples because in the cluster dendrogram, two keratinized samples were located in the non-keratinized group and six non-keratinized samples were in the keratinized group.

Cluster analysis did not lead to the separation of keratinized and non-keratinized oral epithelium samples when only differences in TG3 mRNA levels, or in TG1 and TG3 mRNA levels, were considered (data not shown). On the other hand,

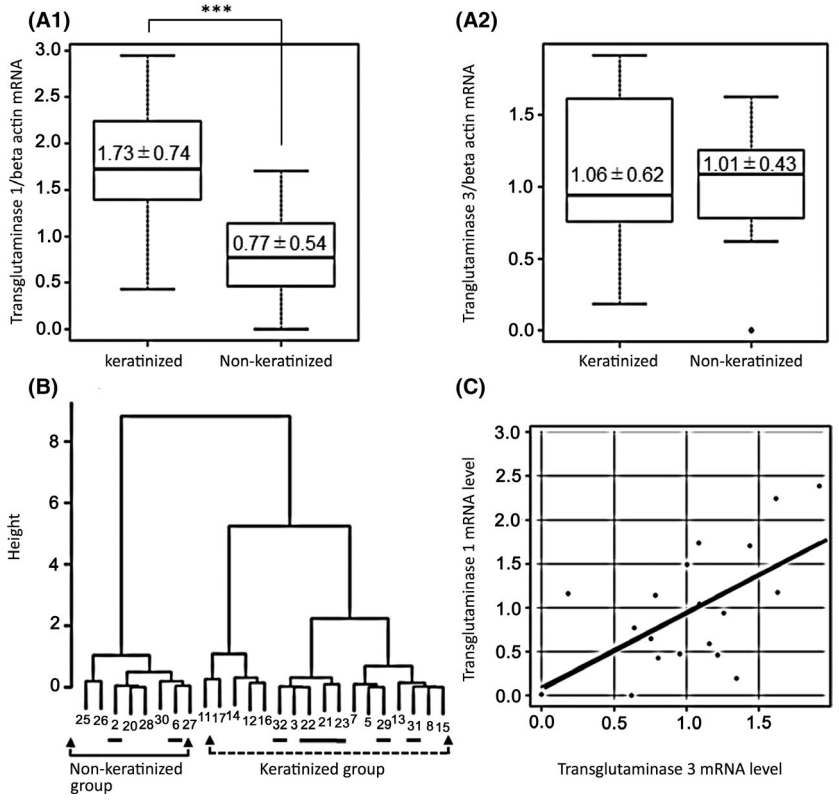

FIGURE 4 Analysis of TG1 and TG3 mRNA levels. Differences in TG1 mRNA (A1) and TG3 mRNA (A2) levels between keratinized oral epithelium (K) and non-keratinized oral epithelium (NK) groups detected by RT-PCR and evaluated by two-sample t-test. Values are presented as mean ±SD; ***$p < 0.005$. Cluster dendrogram based on mRNA levels of TG1 (B). Eight exceptional samples are marked by bars. The non-keratinized group and the keratinized group are indicated by solid and dotted brackets, respectively. Pearson's correlation test highlighted a moderate correlation ($r = +0.595$, $p = 0.007$) between TG1 and TG3 mRNA levels in all keratinized oral epithelium and non-keratinized oral epithelium samples (C).

Pearson's correlation test showed that TG1 and TG3 mRNA levels were moderately correlated in all samples (Figure 4C).

DISCUSSION

The purpose of the present study was to clarify the differences in the distribution and expression of cornified envelope related proteins and genes between keratinized oral epithelium and non-keratinized oral epithelium, and to identify factors that allow to distinguish these two types of epithelium. We demonstrated that keratinized oral epithelium and non-keratinized oral epithelium in the oral mucosa could be separated by cluster analysis considering the following three significant factors: membranous distribution of TG1/TG3/IVL, cytoplasmic distribution of LOR/SPRs, and levels of TG1 mRNA. Therefore, these factors could contribute to cornified envelope formation in keratinized oral epithelium and might be exploited to determine the nature of oral epithelium at different anatomical sites.

We comprehensively demonstrated the difference in TG1/IVL transfer from the cytoplasm to the membrane between keratinized oral epithelium and non-keratinized oral epithelium using these two types of epithelium from different anatomical sites. The complete membranous localization of TG1 could result in the membranous transfer of IVL in the upper spinous layer of many keratinized oral epithelium samples; conversely, incomplete transfer of TG1 retained IVL in the cytoplasm of most non-keratinized oral epithelium samples. These findings are consistent with previous reports in the hard palate (16), gingiva (23), and buccal mucosa (20,24,25). For example, in keratinized oral epithelium mucosa, the localization of TG1 and IVL in the cytomembrane could create favorable conditions for TG1-mediated cross-linking of IVL with other substrates to form the cornified envelope. Similarly, the appearance of TG1 and IVL in cytomembranes facilitated cross-linking of IVL for cornified envelope initiation in epidermal cell lines (6). Therefore, TG1 and IVL localization on membranes could serve as a factor of cornified envelope formation in keratinized oral epithelium. On the other hand, IVL could not translocate from the cytoplasm to the membrane in non-keratinized oral epithelium, thereby possibly inducing the maintenance of a non-keratinized status. However, there is insufficient evidence to explain why in this study two-thirds of non-keratinized oral epithelium samples showed membranous localization of TG1 but lacked IVL transfer to the cell membrane. There are two possible hypotheses to interpret this phenomenon: first, TG1 molecules located in the cell membrane might be in an inactive form; second, the amount of active TG1 might be insufficient for IVL translocation. In any case, lacking or insufficient activity of TG1 caused IVL retention in the cytoplasm, which appears to be an important phenomenon for the determination of the non-keratinizing status.

TG3 was distributed in the cytoplasm of non-keratinized oral epithelium, as previously reported (25). While epidermal TG3 distribution is restricted to the cytoplasm (9,17), our study showed both cytoplasmic and membranous distribution of TG3 in two-thirds of the keratinized oral epithelium samples. To our knowledge, no previous studies have shown TG3 distribution patterns in keratinized oral epithelium, as this analysis was quite beyond their scope. Due to such a lack of knowledge, no consensus has been reached as to why TG3 was found to be localized at the cytomembrane in keratinized oral epithelium. We believe that some TG3 molecules might anchor to the membrane during differentiation of keratinized oral epithelium and thereby contribute to this process via different mechanisms than those involved in the differentiation of the epidermis. Although further studies are needed to clarify this phenomenon, TG3 could mediate a characteristic molecular mechanism of oral epithelium keratinization that is distinct form that of the epidermis.

It is important to note that TG substrates, such as LOR, SPR1b, and SPR3, were not detected in the cornified layer of keratinized oral epithelium but were found in the superficial layer of non-keratinized oral epithelium. However, completely cross-linked cornified envelope component proteins might lose their epitopes and, thus, fail to bind primary antibodies in the cornified layer of keratinized oral epithelium. Our hypothesis is supported by the phenomenon that cross-linking of proteins prevents epitopes from binding antibodies (39), while protein epitopes are exposed after saponification (40). Previous studies have shown that SPR3 is distributed in the palatal, lingual (21), and buccal mucosa (20,21), while LOR is distributed in the palatal mucosa (21) but is absent in non-keratinized oral epithelium, like that of the buccal (21,25) and lingual mucosa (21). Our recent study reported that TG3 and SPRs colocalized with LOR in keratinized oral epithelium, while they localized separately from LOR in non-keratinized oral epithelium. As such, LOR localization is of critical importance for keratinized oral epithelium. We hypothesize that LOR can be cross-linked with SPRs by TG3, strengthening the cornified envelope structure of the cornified layer in the oral mucosa. This hypothesis is supported by the fact that TG3 cross-links LOR with SPRs, reinforcing the cornified envelope scaffold in the epidermis (18).

Several studies have recently addressed transglutaminase gene expression, although very few studies have examined transglutaminase gene expression in the oral mucosa. Our study reports that mRNA levels of TG1 were significantly higher in keratinized oral epithelium than in non-keratinized oral epithelium of the oral mucosa. Similarly, a previous study showed that corneal and conjunctival epithelium undergoing pathological keratinization displayed a significantly higher level of TG1 mRNA than that in non-keratinizing state (41). Contrastingly, in chronic gingival inflammation, TG1 expression was found to be downregulated during regenerative change in tissues in an immature differentiation

state (31). Similar to our results, these studies help explain why the differentiation process involving cornified envelope formation is induced by high levels of TG1 mRNA. Indeed, these findings support our hypothesis that high levels of TG1 mRNA are a factor of cornified envelope formation associated with keratinization in the oral mucosa.

This study has several limitations. First, the primary antibodies against TGs used in our immunohistochemistry experiments could not differentiate active forms of transglutaminases from inactive ones; therefore, there is a need for further studies regarding localization of active transglutaminases that can be evaluated by labelled substrates and sensitivity for cross-linking. Second, there are still unanswered questions concerning the absolute amount and precise distribution of cornified envelope related proteins. Although ethical issues make it difficult to collect enough unfixed tissue samples from voluntary subjects, further investigations by mass spectrographic analysis and electron microscopy are needed. Third, amplification of TG3 cDNA was unsuccessful for two-thirds of keratinized oral epithelium samples because of the potential low quality of nucleic acid; therefore, this limited data failed to yield statistically a significant difference. However, the samples in which transglutaminase genes failed to be amplified by RT-PCR showed positive staining in immunohistochemistry. It is conceivable that proteins could be maintained in tissues, but mRNA may have been degraded due to long-term perseveration as FFPE sample. The small number of samples also limited our research, as it was difficult to obtain normal tissues without inflammation and dysplasia from human surgical specimens. Even though we checked all archived samples in our laboratory, we had to reject a great number of samples after evaluating them for HE, Ki-67, and cytokeratin 19 staining. Therefore, further studies should be conducted using frozen samples or fresh samples.

To the best of our knowledge, this is the first comprehensive study to examine the localization of the main cornified envelope related proteins, including TGs and their substrates, within oral anatomical elements, such as the palate, gingiva, buccal mucosa, and lateral tongue. The characteristics of keratinized oral epithelium differed from those of non-keratinized oral epithelium, with significant differences in the distribution of cornified envelope components and transglutaminase mRNA levels. The results of our study suggest that membranous distribution of TG1/IVL, cytoplasmic distribution of LOR/SPRs, and high levels of TG1 mRNA are factors of cornified envelope formation in keratinized oral epithelium. In addition, TG3 could be a characteristic factor of cornified envelope formation or differentiation of the oral epithelium.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

KS and HH designed this study; RRR and KS retrieved the cases and selected adequate cases, and performed immunohistochemistry, qPCR and statistical analysis; the interpretation of the data was performed by RRR, KS, SM and HH; the manuscript was written by RRR and KS, and edited by HH; all authors reviewed and approved the final version of manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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