Buccal cells as a non-invasive model for filaggrin protein expression and degradation in patients with atopic dermatitis

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Abstract. Mutations in the filaggrin (*FLG*) gene are recognized as major genetic predispositions for atopic dermatitis (AD), yet characterization of FLG protein expression in human subjects remains limited due to the invasive nature of skin biopsies. The present study aimed to evaluate buccal cells as a non-invasive alternative for assessing FLG protein expression and degradation profiles in AD. Buccal cells were collected from 36 patients with AD and 36 healthy controls (aged 20-40 years). *FLG* gene expression was evaluated using reverse transcription quantitative PCR, while FLG protein expression was assessed by immunoblotting. Although *FLG* gene expression exhibited substantial variability, FLG protein levels remained relatively stable across samples. Notably, distinct FLG degradation profiles were observed in patients with AD, particularly involving low-molecular-weight fragments. Mild palmar hyperlinearity was identified in 11% of controls and 33% of patients with AD. In addition, buccal cell pellets from patients with AD exhibited increased viscosity compared with those from controls, which was not explained by mucin expression. These findings suggest that buccal cells provide a feasible, non-invasive epithelial model for investigating FLG protein dynamics, offering a complementary approach to skin-based studies. The observed alterations in degradation profiles and pellet viscosity offer new insights into epithelial barrier dysfunction and support the potential of protein-level approaches in AD research.

Introduction

Filaggrin (FLG) is a key protein in maintaining the integrity and function of the skin barrier; it facilitates the aggregation of keratin filaments within keratinocytes, contributing to the formation of flattened, nucleus-free cells in the stratum corneum (1,2). Processing of profilaggrin by enzymes such as stratum corneum serine protease, peptidylarginine deiminase and caspase-14 generates natural moisturizing factors (NMFs), which are crucial for skin hydration and flexibility (1,3). Mutations in *FLG* are strongly associated with dermatological conditions such as atopic dermatitis (AD) and ichthyosis vulgaris, compromising the skin barrier and increasing susceptibility to environmental irritants and allergens (4-6). Palmar hyperlinearity (PH), characterized by pronounced dermatoglyphics on the palms, has been closely linked with *FLG* null mutations and can serve as a potential phenotypic marker (7,8).

Despite extensive genetic characterization, direct analysis of FLG protein in human samples remains challenging. The complex and highly repetitive structure of *FLG*, particularly exon 3, complicates comprehensive sequencing and limits routine genetic studies. Additionally, obtaining *in vivo* FLG protein data is difficult due to the invasive nature of a skin biopsy, which raises practical and ethical concerns. These limitations highlight the need for alternative approaches to study FLG protein biology and its functional consequences in humans (9).

The buccal mucosa, composed of stratified non-keratinizing epithelium in the inner lining of the cheeks, provides an accessible and non-invasive source of epithelial cells for molecular studies. Previous studies have suggested that buccal cells can serve as a source for mRNA and protein analysis (10-17). However, comprehensive characterization of FLG protein expression and degradation in the context of AD remains limited, particularly regarding disease-specific alterations and functional consequences. Given the practical limitations of obtaining skin biopsies, the present study sought to evaluate whether buccal cells could provide a feasible non-invasive platform for investigating FLG protein expression and degradation in humans.

The present study investigated FLG protein expression and degradation profiles in buccal cells from patients with AD, alongside associated phenotypic features such as PH and cell pellet viscosity. The aim of the study was to evaluate the potential of buccal cells as a non-invasive system for studying FLG protein biology, providing insights into epithelial barrier function and offering a practical alternative to skin biopsies for molecular and diagnostic research.

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Patients and methods

Study participants. The study included individuals aged 20-40 years who were non-smokers and reported no drug use during the study period. Patients with AD (n=36; mean age ± SD, 23.3±6.5 years) and healthy controls (n=36; mean age ± SD, 21.9±4.3 years) were recruited from the student and staff population at Wenzhou-Kean University (Wenzhou, China) between September 2023 and May 2024 through voluntary responses to campus-wide announcements and personal referrals. Inclusion criteria for the patients with AD included having a clear medical record of the disease. Healthy controls had no history of AD, psoriasis or other skin conditions, and did not have a family history of AD. All participants (n=72) were included in phenotypic analyses. Reverse transcription-quantitative PCR (RT-qPCR) analysis was performed in four representative participants (2 controls and 2 patients with AD) to assess expression stability over time. Protein analysis by immunoblotting was performed on samples from 14 healthy controls and 26 patients with AD. Participant allocation across molecular analyses is summarized in Fig. S1.

Buccal cell sample collection. At the time of buccal cell collection, a thorough examination was conducted to confirm that participants did not have any oral mucosal diseases, such as oral lesions or ulcers. Participants thoroughly rinsed their mouths with water and buccal cells were collected by gently chewing the inner cheek with 15 ml of sterile water, followed by spitting into a 50-ml tube. Centrifugation was performed using a Sorvall™ ST 16 Centrifuge (Thermo Fisher Scientific, Inc.) equipped with a F15-6x100y Fixed Angle Rotor (Thermo Fisher Scientific, Inc.). To ensure complete sedimentation, samples were centrifuged at 10,956 x g (10,000 rpm) for 10 min at 4˚C. The pellets were then transferred to 1.5-ml tubes and centrifuged again at 21,100 x g (14,800 rpm) for 10 min at 4˚C using a FRESCO 21 centrifuge (Thermo Fisher Scientific, Inc.). It is noteworthy that mouth rinsing with a commercial oral rinse solution, as opposed to water, resulted in increased viscosity, thereby exacerbating the challenge of separating cell sediments from the supernatant. Pellet viscosity was assessed visually based on pellet edge clarity and compaction following centrifugation at multiple speeds (986, 3,944, 7,800 and 21,100 x g). Palmar hyperlinearity was evaluated using categorical visual scoring. No formal blinding procedures or objective rheological measurements were performed.

Total RNA isolation and gene expression analysis. Total RNA was isolated using Trizol™ (Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. Following RNA extraction, DNase I (RNase-free; cat. no. D7076; Beyotime Biotechnology) treatment was performed to eliminate potential genomic contamination following the manufacturer's recommendations. Reverse transcription was performed using 1 μg of total RNA with the BeyoRT™ III First Strand cDNA Synthesis Kit (cat. no. D7178; Beyotime Biotechnology) according to the manufacturer's protocol. RT-qPCR was conducted using SYBR Green qPCR Mix on a QuantStudio™ 6 Flex system (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. A melting curve analysis was performed to verify amplicon specificity. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (18) and normalized to *GAPDH* as the internal reference gene.

To ensure reliable expression analysis, three primer sets were tested targeting the 5'-terminal region (PrimerBank 60097901c1, 5'-TGAAGCCTATGACACCACTGA-3' and 5'-TCCCCTACGCTTTCTTGTCCT-3'), 3'-terminal region (5'-TGAGGGCACTGAAAGGCAAA-3' and 5'-TGGCCACATAAACCTGGGTC-3'), and highly conserved internal repeat region (5'-CAGACACACARTCAGTGTCAG-3' and 5'-CAYGAATGGTGTCCTGACC-3') of *FLG*. However, only the 5' region primer set met the requirements for RT-qPCR analysis. *FLG* gene expression levels were normalized to *GAPDH* (PrimerBank 378404907c1, 5'-GGAGCGAGATCCCTCCAAAAT-3' and 5'-GGCTGTTGTCATACTTCTCATGG-3'). Gene expression was assessed in 4 representative participants (2 controls and 2 patients with AD). However, *FLG* mRNA levels showed substantial inter- and intra-individual variability across different collection time points (Fig. S2), with Cq values exhibiting poor reproducibility. Given this high variability and the exploratory nature of this analysis, qPCR was not extended to the full cohort, and subsequent investigations focused on protein-level characterization. Biological replicates (batch 1-batch 4) were collected over different months (September, October, November and December 2023) to assess expression stability.

Protein extraction and immunoblotting. For protein extraction, various buffer formulations with modified concentrations of detergents such as SDS, NP-40 and sodium deoxycholate were tested. Despite these efforts, none of the attempts resulted in satisfactory results. Direct cell lysis with SDS loading buffer was found to yield the highest quality results, with successful FLG protein detection in all samples tested. Briefly, buccal cells were collected, and the supernatant was carefully removed. For every 100 mg of cell pellet, 1 ml of 2X SDS loading buffer [25 mM Tris (pH 6.8), 2.5% SDS, 2% glycerol, 4% β-mercaptoethanol and 0.001% bromophenol blue] was added. The pellet was resuspended, boiled for 10 min, cooled on ice, and subsequently centrifuged at 21,100 x g (14,800 rpm) for 10 min at 4˚C.

For western blotting, 10 μl (for FLG detection) or 15 μl (for mucin detection) per lane of protein samples obtained from the previous steps were applied to 12% gels for FLG, 10% gels for mucin (MUC)1, MUC4, MUC7 and MUC16, and 7.5% gels for MUC5B and MUC19, and underwent SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Pall Corporation). Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h and washed with TBST (three times for 5 min each) between all incubation steps.

FLG was detected using two widely used antibodies: mouse anti-FLG antibody AKH1 (1:750, cat. no. sc-66192, Santa Cruz) and rabbit anti-FLG antibody Poly19058 (1:750; cat. no. 905804; BioLegend, Inc., raised against residues 24-39 of FLG). Membranes were first probed with AKH1 in blocking buffer overnight at 4˚C, followed by the appropriate

IRDye-conjugated secondary antibody at room temperature for 1 h, and visualized using the ODYSSEY CLx imaging system (LI-COR Biosciences). The same membranes were then probed with Poly19058 overnight at 4˚C, followed by the appropriate secondary antibody at room temperature for 1 h, and visualized again. This sequential probing strategy took advantage of the dual-channel capacity of the Odyssey system. To confirm protein loading, membranes were then stripped with NewBlot™ IR Stripping Buffer (cat. no. 928-40028; LI-COR Biosciences) according to the manufacturer's protocol. Complete signal removal was confirmed by re-scanning prior to reprobing. The stripped membranes were blocked again with 5% BSA in TBST at room temperature for 1 h and probed with rabbit anti-keratin 14 (KRT14) antibody (1:1,000; cat. no. A19039; ABclonal Biotech Co., Ltd.; KRT14) overnight at 4˚C, followed by goat anti-rabbit IRDye 800CW secondary antibody at room temperature for 1 h. KRT14 was selected as a loading control for FLG and mucin blots due to its stable expression as a structural protein in epithelial cells and as it is well separated from potential FLG monomer fragments. This selection follows established practice in the FLG field (6,19) and has been used in subsequent studies of FLG expression in epithelial tissues (20,21). For mucin detection, membranes transferred from 10% SDS-PAGE gels were probed sequentially with rabbit anti-MUC4 antibody (1:750; cat. no. A3438; ABclonal Biotech Co., Ltd.), rabbit anti-MUC7 antibody (1:1 000; cat. no. 26544-1-AP; Proteintech Group, Inc.), and, after stripping, mouse anti-MUC1 antibody (1:250; cat. no. sc-7313; Santa Cruz Biotechnology, Inc.) and rabbit anti-MUC16 antibody (1:500; cat. no. A4666; ABclonal Biotech Co., Ltd.). Membranes transferred from 7.5% SDS-PAGE gels were probed with mouse anti-MUC5B antibody (1:100; cat. no. sc-21768; Santa Cruz Biotechnology, Inc.) and rabbit anti-MUC19 antibody (1:500; cat. no. bs-17905R; BIOSS) under identical conditions as aforementioned. Secondary antibodies used were goat anti-mouse IRDye 680RD (cat. no. 926-68070; LI-COR Biosciences) and goat anti-rabbit IRDye 800CW (cat. no. 926-32211; LI-COR Biosciences), both at 1:15,000 dilution. Membranes were washed with TBST three times for 5 min after each antibody incubation.

Statistical analysis. Data were analyzed using GraphPad Prism (v8.0; Dotmatics). For categorical data in Fig. 1D, comparisons between healthy controls and patients with AD were performed using the two-tailed Fisher's exact test. $P<0.05$ was considered to indicate a statistically significant difference. No formal statistical comparisons were performed on the immunoblotting data (Figs. 2, S3 and S4) and the results are presented descriptively.

Results

Phenotypic manifestations of palmar hyperlinearity and buccal cell pellet viscosity in participants with AD. In the present study, 36 patients with AD and 36 healthy controls, aged 20-40 years, were recruited. Initial exploratory analyses, including phenotype observation, gene expression analysis and protein profiling, were performed in 2 healthy controls (H1 and H2) and 2 patients with AD (AD1 and AD2), with AD2 reporting a family history of AD.

PH, characterized by prominent palmar creases, is frequently associated with *FLG* deficiency (7,8). Among the representative participants, H2 and AD1 showed no evident PH, H1 exhibited mild PH, and AD2 displayed a pronounced PH phenotype (Fig. 1A and B). Notably, during buccal cell collection, a marked difference in pellet properties was observed between groups. Samples from the patients with AD consistently exhibited increased viscosity and poorly defined pellet boundaries, whereas control samples formed compact pellets with sharp edges (Fig. 1B and C). Even at high centrifugal force, AD-derived pellets remained diffuse and difficult to compact. Across the full cohort, mild PH was observed in 11% of healthy controls compared with 33% of patients with AD (Fig. 1D). Similarly, increased pellet viscosity was more frequently observed in AD samples at all tested centrifugation conditions.

To investigate potential contributors to this phenotype, mucin expression was assessed; however, no marked or consistent differences were observed between the AD and control groups (Fig. S3), suggesting that mucins are unlikely to account for the increased viscosity.

Limited consistency of FLG gene expression in buccal cells. *FLG* gene expression was evaluated in a subset of participants (H1, H2, AD1, and AD2) across four independent collections over different months (Fig. S2). Expression levels were detectable in all samples but exhibited substantial intra- and inter-individual variability over time. Given the limited sample size and exploratory nature of this analysis, these data are presented descriptively to illustrate variability rather than for statistical inference. Subsequent analyses therefore focused on protein-level characterization.

Stable FLG protein expression with altered degradation profiles in AD samples. To investigate FLG protein expression, two widely cited antibodies were utilized for FLG protein detection: AKH1 (mouse monoclonal) and Poly19058 (rabbit polyclonal, raised against residues 24-39 of filaggrin) (6,22-25). Across all participants, signals corresponding to putative FLG-derived fragments were consistently detected. By contrast, distinct differences in FLG degradation profiles were observed between patients with AD and healthy controls (Fig. 2). Notably, low-molecular-weight fragments, particularly around ~8 kDa, showed clear differences between groups and were consistently detected by both antibodies. Additional variability was observed in intermediate fragments (20-40 kDa), suggesting altered processing or degradation of FLG in AD samples.

Analysis of an expanded cohort (Fig. S4; Table SI) confirmed that FLG degradation profiles differed between patients with AD and healthy controls. Notably, the putative terminal ≤10 kDa fragment was less frequently detected in AD samples (19% with AKH1 and 4% with Poly19058) than in the healthy control samples (50% with both antibodies), while greater diversity in the 20-40 kDa region was observed in a subset of AD samples. Together, these findings indicate that while overall FLG protein expression remains stable in buccal cells, its degradation profile is altered in AD.

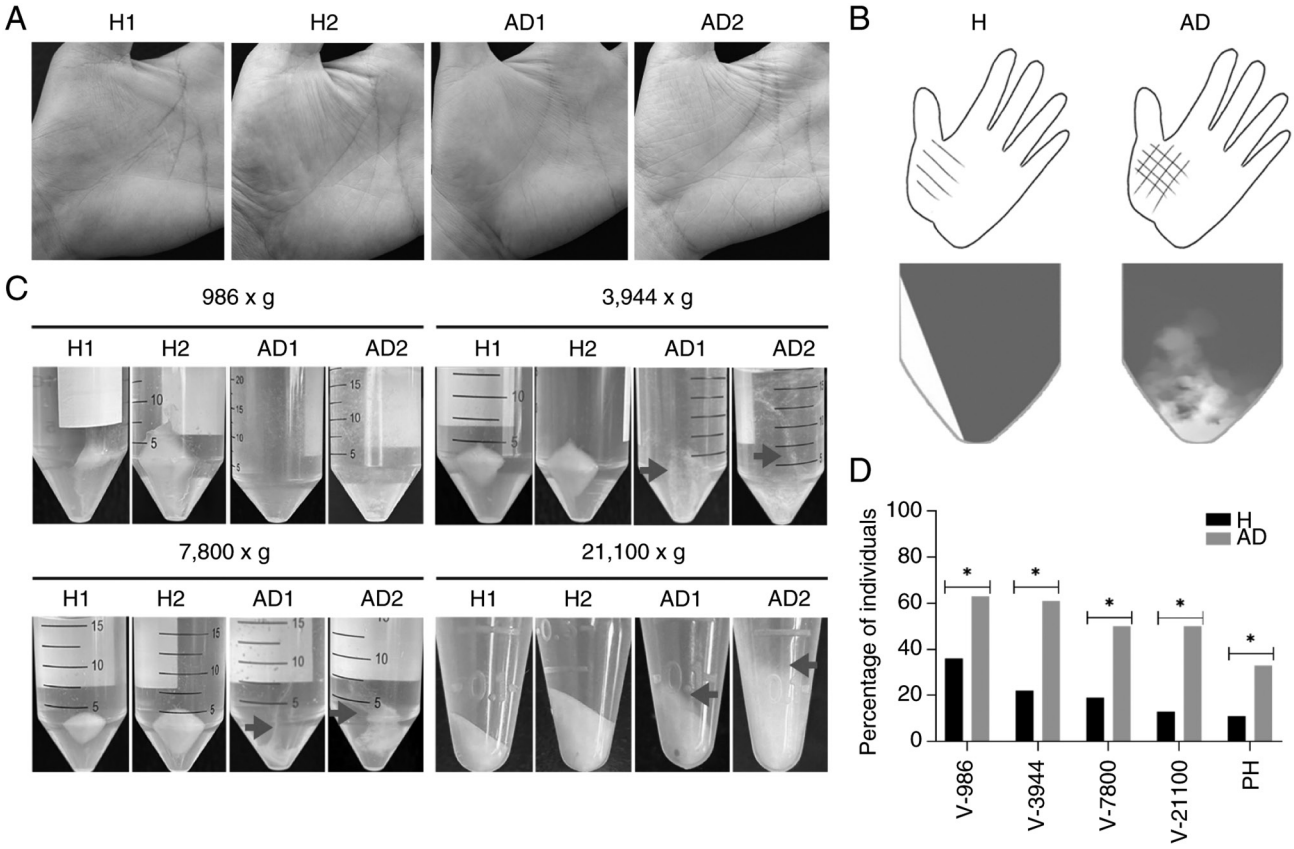

Figure 1. Phenotypic differences between healthy controls and patients with AD. (A) Palm prints of four participants showing phenotypic variations. (B) Schematic representation of the observed phenotypes. The images at the bottom represent centrifugation tubes with cell pellets. (C) Phenotypic variations in buccal cell pellets after centrifugation at different speeds for 2 min. Healthy controls (H1 and H2) display tightly packed and clearly marginated pellets, while patients with AD (AD1 and AD2) show loose and fluffy pellets with unclear edges, even after centrifugation at 21,100 x g for 10 min. Arrows highlight areas with distinct phenotypic differences. (D) Quantitative analysis of PH and buccal cell pellet viscosity in 36 healthy controls and 36 patients with AD. 'V-986', 'V-3944', 'V-7800' and 'V-21100' indicate viscosity at corresponding centrifugation speeds (x g). *P<0.05, as determined using a two-tailed Fisher's exact test. H, healthy control; AD, atopic dermatitis; PH, palmar hyperlinearity.

Discussion

FLG stands as the most robustly identified genetic predisposition for AD (1,5,19,26), yet investigations into FLG protein expression among patients with AD remain scarce due to challenges in biopsy acquisition. Despite nearly two decades of intensive research, most FLG studies have focused on genetics, transcriptomics, immunohistochemistry, animal models or cell culture systems (1,2,6,19). By contrast, direct FLG protein data obtained from human tissues remain limited, and only a small number of studies have successfully performed western blot analysis of FLG proteins in human samples (6,19). This long-standing technical challenge has hindered efforts to characterize FLG processing and degradation *in vivo*. The present study demonstrates that buccal cells provide a feasible and non-invasive system for assessing FLG protein expression and degradation profiles in humans.

Following extensive optimization of protein extraction methods, a broad spectrum of FLG-immunoreactive species was reproducibly detected in human buccal cells using two independent antibodies recognizing distinct epitopes. While *FLG* mRNA levels showed variability across samples and time points, distinct differences in FLG degradation profiles were observed between patients with AD and healthy controls, characterized by reduced detection of the terminal ≤10-kDa fragment and greater diversity in the intermediate fragment range in AD samples. This pattern points to potential differences in FLG processing or catabolism in AD, extending beyond simple changes in overall expression levels. Given the central role of FLG degradation in generating NMFs, such alterations may have functional implications for epithelial barrier properties and warrant further investigation using complementary approaches such as mass spectrometry and genetic characterization.

In addition to molecular findings, a reproducible increase in buccal cell pellet viscosity was observed in patients with AD. This phenotype was not explained by differences in mucin expression, suggesting that other factors may contribute to this effect. Several hypotheses may explain the increased viscosity observed in AD buccal cell pellets. One possibility is that FLG degradation products, which are abundant in epithelial cells and show altered hydrolysis profiles in AD, may directly influence the biochemical and physical properties of the cellular pellet (27,28). Another is that impaired epithelial barrier function and altered tissue hydration in AD could affect cell-cell adhesion or pellet compaction during centrifugation (29,30). A third possibility is that differences in the composition of intracellular proteins, extracellular matrix components or inflammatory exudates may also contribute to this phenotype (31,32). Although

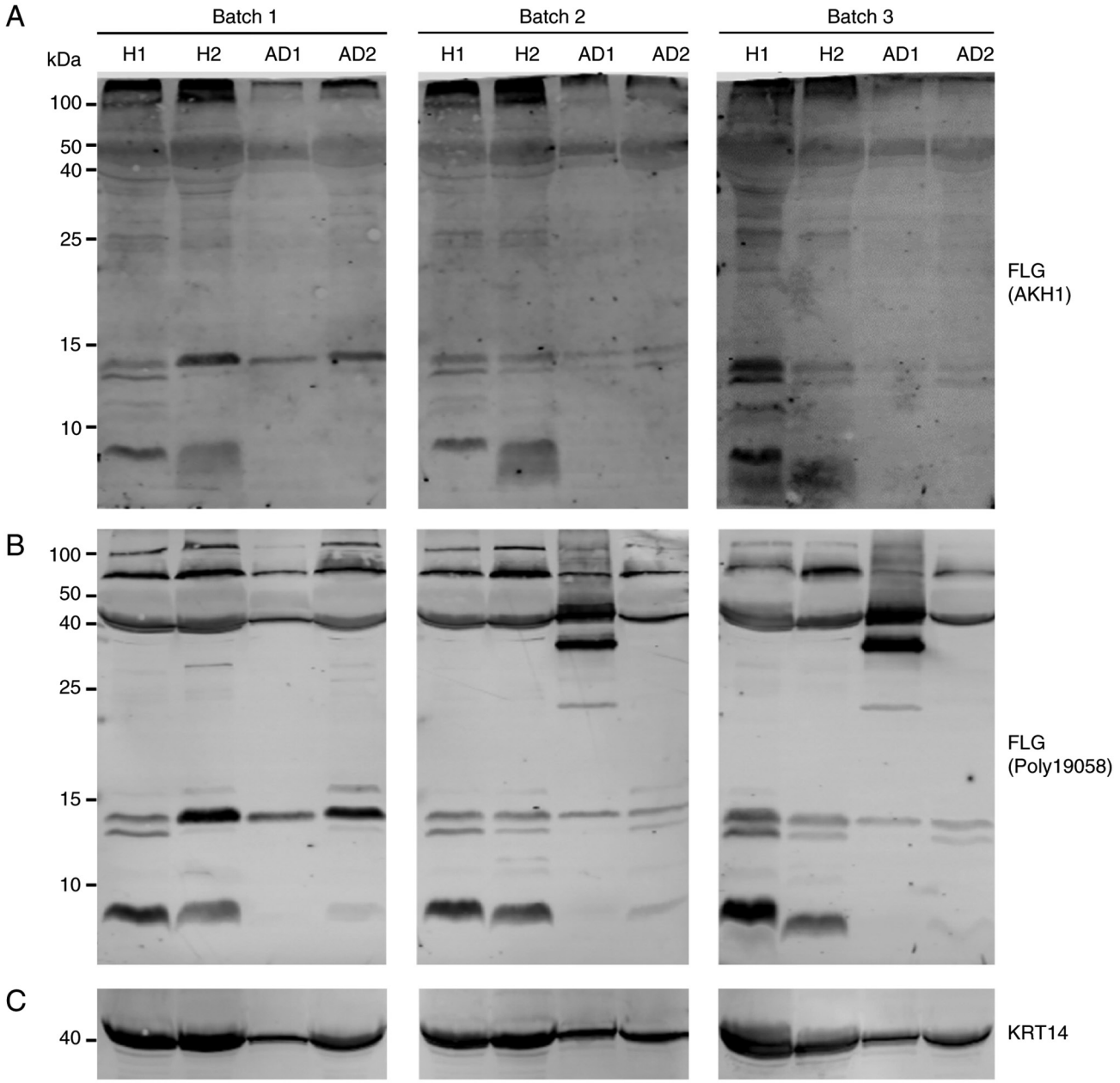

Figure 2. Expression of FLG in buccal cells. (A) Immunoblotting with mouse anti-FLG monoclonal antibody AKH1 demonstrates stable expression of FLG protein in buccal cells, with major bands observed at positions ~100, ~50, ~40, ~25, ~15 and ~8 kDa. (B) Immunoblotting with rabbit anti-FLG polyclonal antibody Poly19058 also reveals stable expression of FLG protein in buccal cells, with major bands detected at positions ~100, ~70, ~40, ~15, and ~8 kDa. (C) KRT14 was used as a protein loading control. Representative immunoblots of four participants (H1, H2, AD1 and AD2) are shown; expanded cohort data are presented in Fig. S4. H, healthy control; AD, atopic dermatitis; FLG, filaggrin; KRT14, keratin 14.

speculative, these possibilities highlight a previously underappreciated aspect of epithelial biology in AD that warrants further investigation.

Several limitations should be acknowledged. First, due to the non-invasive study design, direct comparisons between buccal and skin tissues were not performed. Therefore, buccal cells should be considered a complementary system rather than a direct substitute for epidermal tissue. Second, comprehensive genotyping was not conducted, as the highly repetitive structure of the *FLG* gene presents substantial technical challenges. Furthermore, loss-of-function mutations in *FLG* are rare in East Asian populations, making genotype-phenotype correlation difficult even with targeted screening in a cohort of this size. Third, standardized disease-severity assessments such as Scoring Atopic Dermatitis tool (33) or the Eczema Area and Severity Index (34) were not available for the participants in this study. Fourth, the protein analyses were primarily qualitative, and densitometric semi-quantification was not performed. In addition, definitive identification of the observed immunoreactive bands was not established by orthogonal methods such as mass spectrometry; however, the detection of the ~8-kDa signal by two independent antibodies recognizing distinct epitopes provides cross-validation of its FLG-derived origin. These signals are therefore referred to as putative FLG-derived fragments. Fifth, phenotypic assessments, including PH and pellet viscosity, were evaluated

using categorical visual scoring without formal blinding or objective rheological measurement. Future studies incorporating quantitative protein analysis, genetic characterization and clinical severity scoring will be necessary to further elucidate FLG regulation and its association with disease activity.

In conclusion, the present study establishes buccal cells as a practical and accessible model for investigating FLG protein biology in humans. The identification of altered degradation profiles and increased pellet viscosity in AD provides new insights into epithelial barrier dysfunction and supports the potential of non-invasive approaches for studying complex skin diseases.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

KL conceived and designed the study, performed data curation, methodology development and investigation, and drafted the manuscript. XZhou and XZou performed data curation, methodology development and investigation. SJ, ZG, XZhu, ZZ and YL contributed to data curation and investigation. BZ conceived and supervised the study, contributed to methodology, project administration, funding acquisition, and manuscript writing and revision. All authors have read and approved the final manuscript. KL and BZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study protocol was approved by the Ethics Committee of Wenzhou-Kean University (Wenzhou, China; approval nos. WKU20210002 and WKUIRB2022017) and all methods were conducted following the principles of the Declaration of Helsinki. All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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