8-1-2016

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HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2017 August 01.

Published in final edited form as:

J Invest Dermatol. 2016 August ; 136(8): 1732–1735. doi:10.1016/j.jid.2016.03.037.

Quantitative proteomic analysis of Stratum Corneum dysfunction in adult chronic Atopic Dermatitis

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To the Editor

Atopic Dermatits/Eczema (AD) is a common condition in childhood and is also suffered by approximately 2–3% of adults (Eichenfield et al., 2014). Despite intense scientific investigation into the genetics of AD, disease-linked loci are associated with a minority of disease cases (Baurecht et al., 2015), leaving the etiology of spontaneous AD in question.

To better understand the biology of AD and to identify protein markers of disease, we applied quantitative techniques to identify differential Stratum Corneum (SC) proteins from adult AD patients and normal individuals. Using discovery proteomics we identified over 1000 proteins in the SC, with over 200 differentially identified by condition. Selected proteins were quantified with precise targeted methods. Technical details are described in the Supplementary Materials and Methods (online).

Specifically, we profiled skin surface tape strip samples from 11 AD subjects at lesional and nonlesional sites. These were compared with similar samples from 17 healthy subjects. Combining all spectral evidence, we identified 1102 proteins at a 1% false positive error rate (FPER). This represents the most comprehensive proteomic study of AD stratum corneum to date (Broccardo et al., 2011, Sakabe et al., 2014). The full identification list can be found in Supplementary File S1.

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Conflict of Interest

This research was funded in part by Procter and Gamble (P&G) as part of a general collaboration between P&G and the Institute for Systems Biology. DF, KJM, TH, CCB and RJI are all employees of P&G and contributed to study design, data collection and analysis.

Statement of Informed Consent

All research was carried out under the Declaration of Helsinki protocols, written informed consent was obtained from all individuals, and experiments were approved by the Western Institutional Review Board (Study number 1121688).

Work performed in Seattle, WA, USA and Cincinnati, Ohio, USA.

A poorly characterized aspect of skin biology is the role of protein post-translational modifications which are abundant in the outer layers of the epidermis. One such is modification is citrullination (conversion of arginine to citrulline, also called deimination) (Gyorgy et al., 2006). We included citrullination in our search parameters and filtered the results to avoid false positives. We detected at least one citrullination site on 1005 proteins (91% of those identified). The most citrullinated proteins were trichohyalin and filaggrin, which are known to be heavily modified *in vivo*. For filaggrin, healthy and nonlesional atopic skin were citrullinated on 78% and 75% of arginine residues, respectively. This decreased to 62% in lesional skin, corresponding to a loss of modification on 56 residues. Of the 386 proteins that were citrullinated in all conditions, 257 exhibit decreased fractional citrullination in lesional sites compared to other conditions (Figure 1). Notably, trichohyalin, an abundant hair protein not considered related to AD, showed a negligible difference by the same comparison.

We next analyzed differential proteins by two methods: Fisher's exact test on identification counts by condition and Student's *t*-test on label-free quantification results for proteins observed in greater than 10 samples (Figure 2A & 2B, repectively). 252 proteins were differentially observed between healthy and AD samples (Figure 2A & Supplementary File S1). Many were general plasma-related markers of inflammation. Preferentially observed in AD included proteins related to inflammation and barrier function such as serpins A3, B4, & B5, interleukin-1 receptor antagonist, interleukin-36 receptor antagonist, protein disulfide isomerases, and S100s A4 & A6. In addition, 36 proteins were found as differentially abundant via *t*-test (Figure 2B). These include inflammation-related proteins such as serpins A1, B3, & B12 and S100s A8, A9, and A11. Proteins down-regulated in AD skin are generally structural, including several keratins as well as filaggrin-2. Fillagrin-2 was only annotated in 2009 (Wu et al., 2009) and mutations have been recently linked to persistent AD (Margolis et al., 2014). When comparing nonlesional and lesional AD samples, 45 proteins are differentially observed (Figure 2A & Supplementary File S1). 16 are preferentially found at the lesional site while 29 are observed more frequently in nonlesional samples. The protein most preferentially identified at the lesional site was PYCARD, a key regulator of inflammasome and activation and apoptosis (Martinon et al., 2002). One protein found at the majority of nonlesional sites but only at 9% of lesional sites was SPINK5, a gene with mutations linked to AD in Japanese patients (Kato et al., 2003).

To confirm results from discovery LC-MS/MS, we targeted several AD-related proteins using more sensitive and precise Selected Reaction Monitoring (SRM) mass spectrometry-based assays. These results support our shotgun LC-MS/MS label-free quantification, but with increased sensitivity as expected. Caspase 14, beta-defensin, desmoglein-1, desmoplakin, filaggrin, kallikrein-8, keratin 10, S100-A7, and S100-A9 all increase when comparing healthy to atopic nonlesional skin. In addition, beta-defensin, filaggrin, S100-A7, and S100-A9 further increase from nonlesional to lesional atopic skin. The only targeted protein to show a significant decline is filaggrin-2, which decreases substantially from nonlesional compared to lesional epidermis, in agreement with label-free quantification above. Given the evidence for reduced abundance of filaggrin in atopic dermatitis from several studies (Suarez-Farinas et al., 2011, Pellerin et al., 2013), our observation of increased SRM signal in atopic skin may seem counterintuitive. Our label-free quantification

found filaggrin to be similar between healthy and AD (p=0.2, supplementary file S1). It is important to consider that in the targeted SRM assay we are monitoring SRM signal from the fully tryptic unmodified peptide, ^{3976}HGSYGSADYDYGESGFR^{3992}. In addition to the mass shift introduced by citrullination, we observed that this modification typically results in a missed cleavage at arginine, rendering the peptide invisible to the SRM assay deployed here. Thus our observation is consistent with decreased citrullination on the endogenous peptide as disease progresses. Indeed, the majority of evidence for filaggrin protein loss comes from immunohistochemistry experiments where antibody epitopes are not always characterized. It is possible that these epitopes are citrullinated, similar to those observed *in vivo* for rheumatoid arthritis (Girbal-Neuhauser et al., 1999).

While genetic factors tied to AD have been investigated in some detail, these account for a minority of disease incidence (Baurecht and Hotze, 2015). Much focus has been directed toward filaggrin loss-of-function mutations, however transcriptomic profiles stratified by filaggrin mutation status demonstrate an up-regulation of this protein in wild-type AD patients (Cole et al., 2014). We find a modest overlap between transcript and proteomic differential markers (Supplementary information). Non-genetic potential disease factors including microbiome diversity and epigenetic factors like DNA methylation have only recently begun to be investigated (Kong et al., 2012, Rodriguez et al., 2014). The data presented here demonstrate alterations in the protein post-translational modification profile of patients with AD lesions which ultimately adds another dimension to the rich and complex etiology of AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Michael R. Hoopmann and Dr. Ulrike Kusebauch for technical assistance and early access to the Human SRMAtlas. This work was funded in part by the American Recovery and Reinvestment Act (ARRA) funds through National Institutes of Health from the National Human Genome Research Institute grant no. RC2 HG005805; the National Science Foundation MRI Grant No. 0923536, the National Institute of General Medical Sciences under Grant Nos. 2P50 GM076547/Center for Systems Biology, S10RR027584, and GM087221.

Abbreviations

AD	Atopic Dermatitis
FPER	False Positive Error Rate
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
SC	Stratum Corneum
SRM	Selected Reaction Monitoring

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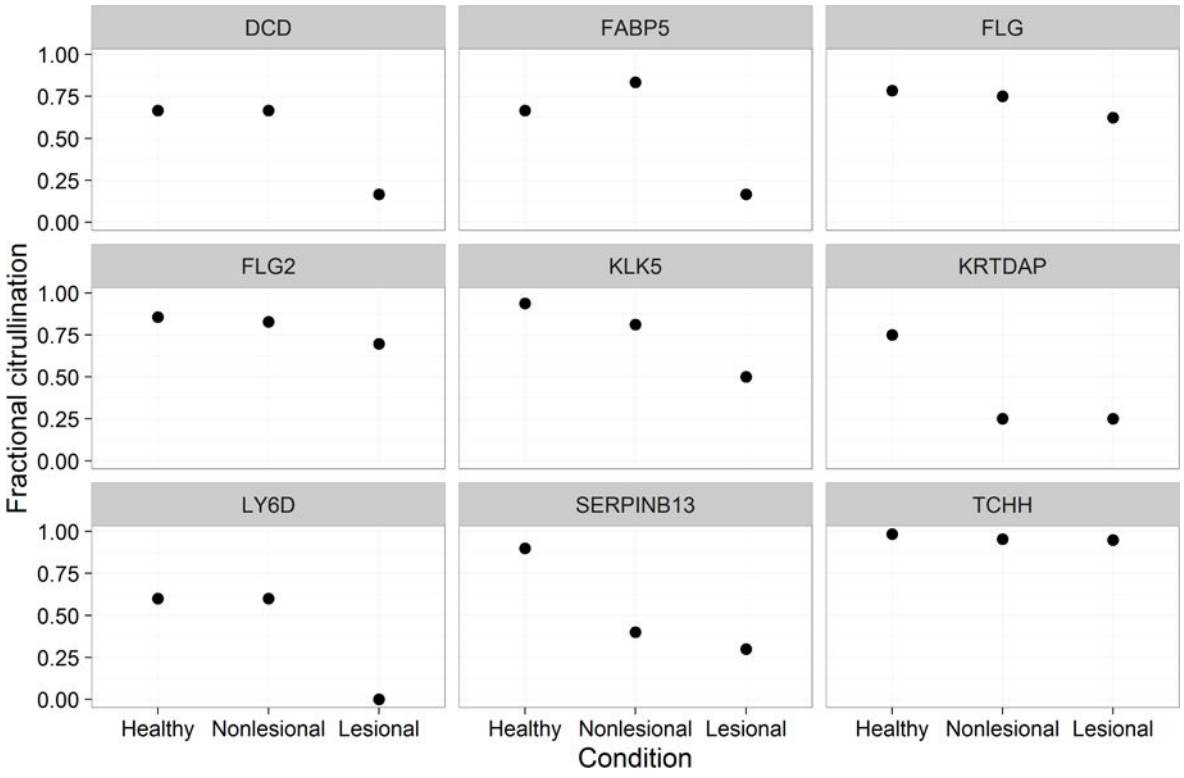

Figure 1.

Fractional citrullination (modifications per arginine) for selected proteins. Many proteins related to epidermal differentiation and homeostasis decrease in citrullination with perturbation. The unrelated but highly citrullinated protein trichohyalin (TCHH) shows little change with condition.

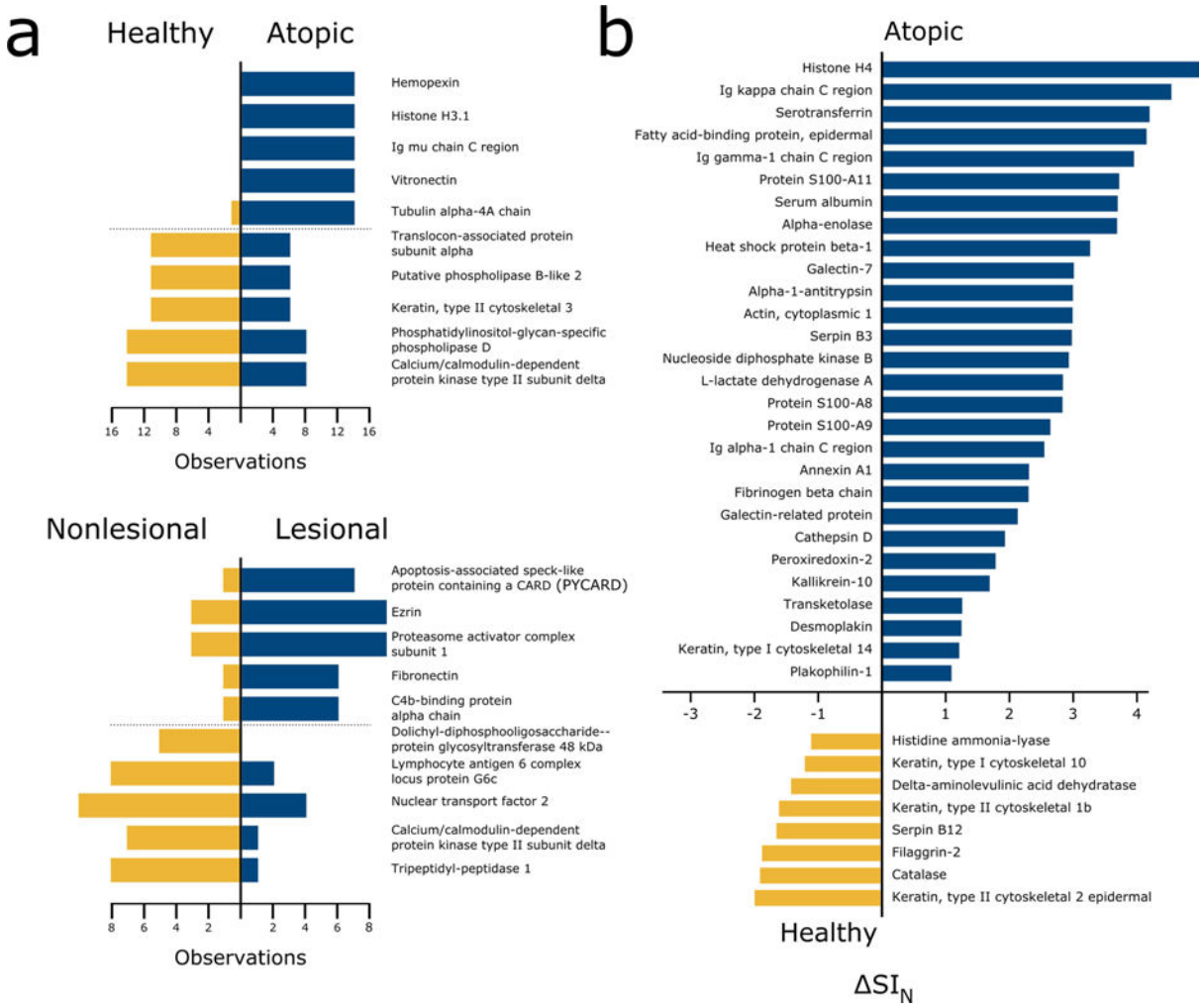

Figure 2.

A) Differentially identified proteins ($p < 0.05$) from Fisher's exact test. Bar lengths indicate the observations by clinical condition. Proteins with the 5 lowest p-values are shown for each condition. The full data table is in Supplementary file S2. B) Differentially abundant proteins (adjusted $p < 0.05$) for healthy vs. atopic (nonlesional and lesional) samples. Bars represent the difference in average Normalized Spectral Index by clinical condition. Positive values indicate higher abundance in atopic samples and negative values indicate higher abundance in healthy samples.