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Serum Ceruloplasmin as a Potential Clinical Biomarker in Atopic Dermatitis

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Background: Although the discovery of new biomarkers in atopic dermatitis (AD) is challenging, it is valuable in diagnosis, assessment of severity, and evaluation of treatment response.
Objective: This study was designed to identify and validate new candidate protein biomarkers of AD via proteomic analysis.
Methods: Comparison of protein expression in the patients' serum before- and after-treatment and in patients without AD was performed via two-dimensional gel electrophoresis (2-DE), followed by image analysis. Spots showing differential expression in 2-DE image analysis were identified subsequently via nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments. Blood tests were conducted to validate the results obtained by measuring the levels of candidate proteins.
Results: Based on LC-MS/MS analysis and database search, we identified ceruloplasmin (Cp) as a candidate protein. Serum Cp levels were significantly decreased in in pre-treated AD group than in control group. Additionally, the serum Cp level was higher in the mild group than in the moderate group of AD based on the Eczema Area and Severity Index (EASI) score.
Conclusion: Low serum Cp levels are associated with AD, suggesting the potential role of Cp as a biomarker for diagnosis and severity assessment of AD.

Keywords: Atopic dermatitis, Ceruloplasmin, Mass spectrometry, Proteomics

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with heterogeneous pathogenesis and diverse clinical manifestations. AD can have a profound effect on quality of life and lead to a huge socioeconomic burden[1]. The etiology of AD is complex, including multiple factors, such as genetic abnormalities, dysregulated skin barrier, immune imbalance, and altered skin microbiome[2-4].

In addition to efforts to identify the etiology of AD, efforts are ongoing to identify reliable biomarkers that not only act as diagnostic tools but determine the severity of the disease and the response to treatment. To date, unlike other chronic diseases, clinicians have largely relied on clinical tools such as Eczema Area and Severity Index (EASI), Investigator's Global Assessment (IGA), and SCORing Atopic Dermatitis (SCORAD) to assess the severity of AD and treatment response[5].

A significant number of biomarkers have been discovered and clinically utilized in the diagnosis of AD and assessment of severity or treatment outcome. However, the efficacy of biomarkers in reflecting the severity of the disease varies drastically. Moreover, most biomarkers cannot be easily evaluated with simple blood test. Instead, it is often necessary to use expensive test kits to analyze blood or tissue samples or even perform immunohistochemical staining using skin biopsy specimens. In this context, efforts are needed to identify serum biomarkers correlating with AD severity and treatment response based on simple blood tests.

In the present study, we conducted a proteomic analysis via two-dimensional gel electrophoresis (2-DE) and nanoscale liq-

uid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the differential expression of proteins before and after treatment of AD. Additionally, in order to validate the protein as a reliable biomarker, the serum levels of the protein were measured in the blood samples of a larger number of disease and control groups and compared according to the severity score.

MATERIALS AND METHODS

Enrollment of study subjects

We enrolled patients with clinically diagnosed AD for the proteomic analysis. AD was diagnosed according to the modified Hanifin and Rajka criteria[6] issued by Korean Atopic Dermatitis Association. The participants were treated with oral cyclosporine or a biologic agent such as dupilumab for more than 16 weeks. We also included healthy volunteers with no history of atopy, allergic diseases, or type I hypersensitivity related disorders. In addition, larger numbers of AD and control groups were recruited for measuring candidate protein biomarker in the serum. This study was approved by the Institutional Review Board of Hallym University Dongtan Sacred Heart Hospital (IRB file No. HDT 2020-06-025), and all participants provided written informed consent.

Clinical and laboratory data collection

We collected subjects' clinical characteristics including age, sex, duration of the AD, accompanying diseases. Severity scores assessed with EASI, IGA, pruritus numerical rating scale (NRS), and Dermatology Life Quality Index (DLQI) were obtained before and after treatment with a systemic agent or dupilumab. The patients were divided into three subgroups based on EASI score (mild: <16; moderate: 16≤ and <23; severe: ≥23) and DLQI score (0~5: zero to small effect on patient's life; 6~10: moderate effect on patient's life; 11~30: large-to-extremely large effect on patient's life). Laboratory data including complete blood count, routine chemistry, serum total immunoglobulin E (IgE) (IU/ml), eosinophil count (/mm^3), and lactate dehydrogenase (LDH) (U/L) level were also measured before and after treatment.

Sample collection and preparation for proteomic analysis

Blood samples were collected from patients and healthy subjects. In addition to conventional blood sampling, additional 3 ml samples of blood were collected into a 5 ml BD vacutainer serum separation tube for the proteomic experiment.

The tubes were allowed to clot for 30 minutes, and then centrifuged at 1,300 ×*g* for 10 minutes in a swinging bucket centrifuge within 2 hours. The separated serum was transferred into 1.5 ml tubes in 500 μl aliquots and stored at –70℃ until further use.

Two-dimensional gel electrophoresis and image analysis

A 2-DE was carried out essentially as described. Aliquots in sample buffer (7 M urea, 2 M thiourea, 4.5% CHAPS, 100 mM DTE, 40 mM Tris, pH 8.8) were added to immobilized pH 3~10 nonlinear gradient strips (Amersham Biosciences, Uppsala, Sweden). Isoelectrofocusing was performed at 80,000 Vh. The second dimension was analyzed on 9%~16% linear gradient polyacrylamide gels (18 cm×20 cm×1.5 mm) at constant 40 mA per gel for approximately 5 hours. After protein fixation in 40% methanol and 5% phosphoric acid for 1 hour, the gels were stained with coomassie brilliant blue G-250 for 12 hours. The gels were destained with H_2O, scanned in a Bio-Rad (Richmond, CA, USA) GS710 densitometer and converted to electronic files, which were then analyzed with an Image Master Platinum 5.0 image analysis program (Amersham Biosciences).

LC-MS/MS analysis of peptides

LC-MS/MS analysis was performed with an Easy n-LC (Thermo Fisher, San Jose, CA, USA) and a LTQ Orbitrap XL mass spectrometer (Thermo Fisher) equipped with a nano-electrospray source. Samples were separated on a C18 nanobore column (150 mm×0.1 mm, 3 μm pore size; Agilent, Santa Clara, CA, USA). The mobile phase A for LC separation included 0.1% formic acid and 3% acetonitrile in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 0% B to 60% B in 9 minutes, 60% B to 90% B in 1 minute, and 3% B in 5 minutes. The flow rate was maintained at 1,800 nl/min.

Mass spectra were acquired using data-dependent acquisition with a full mass scan (380~1,700 m/z) followed by 10 MS/MS scans. The orbitrap resolution for MS1 full scans was 15,000 and the automatic gain control (AGC) was 2×10^5. For MS/MS in the LTQ, the AGC was 1×10^4.

Database search

The mascot algorithm (Matrixscience, USA) was used to identify peptide sequences in a protein sequence database. Database search parametes were: *Homo sapiens*; *Homo sapiens*,

fixed modification; carbamidomethylated at cysteine residues, variable modification; oxidized at methionine residues, maximum allowed missed cleavage; 2, mass-spectrometry (MS) tolerance; 10 ppm, MS/MS tolerance; 0.8 Da. The peptides were filtered with a significance threshold of $p<0.05$.

Statistical analysis

Data were expressed as mean±standard deviation. Statistical analyses were performed using IBM SPSS statistics ver. 21 (IBM Corp., Armonk, NY, USA). Statistical analyses were done using Student's t-test, one-way ANOVA followed by multiple comparisons with Tukey's honestly significant difference test. A *p*-value of less than 0.05 was considered significant.

RESULTS

Characteristics of study subjects

The general characteristics of the study subjects are presented in Table 1. Among a total of 86 subjects (44 males and 42 females; mean age 28.51±5.46 years), 31 subjects manifested AD and 55 subjects were healthy volunteers. The proportion of sex and age was similar between the two groups. The AD group was divided into pre-treatment and post-treatment subgroups. Most patients received the two medications sequentially. However, 6 patients received oral cyclosporine alone and 4 patients received only dupilumab. The mean EASI, IGA, pruritus NRS, and DLQI scores of the two groups were 18.04, 1.65, 4.97, and 11.02, respectively, which suggested a statistically significant difference. However, there were no significant differences in the levels of serum total IgE, eosinophil count, and LDH level between two groups.

Results of 2-DE and image comparison between control, pre- and post-treatment groups

Sera obtained from eight control subjects (four males and four females; mean age 25.6±5.6 years) and eight AD subjects (four males and four females; mean age 27.3±4.9 years) were analyzed via 2-DE. Samples selected from the AD group were pooled pre-treatment and post-treatment from the same participants. The difference in protein expression between the three groups was compared via 2-DE based on at least 2.5-fold difference between pre and post-treated groups and between pre-treated and control groups. Differential protein expression was observed in 12 spots (Fig. 1) including four spots over-expressed and eight under-expressed in the pre-treatment group.

Identification of differentially expressed proteins

The 12 spots analyzed via 2-DE and subsequent image comparison were subjected to LC-MS/MS analysis for protein identification (Table 2). Ceruloplasmin (Cp; protein accession number gi|116117) was identified as an under-expressed protein in the pre-treatment group compared with both control and post-treatment groups.

Table 1. Clinical characteristics of study subjects

Characteristic	Control (n=55)	Atopic dermatitis		*p*-value*
		Pre-treatment (n=31)	Post-treatment (n=31)	
Age (yr)	28.93±5.40	27.77±6.45		NS
Male (%)	28 (50.9)	16 (51.6)		NS
Disease duration (yr)	-	12.32±5.5		-
Mean treatment duration (wk)	-	14.87±1.6		-
Total IgE (IU/ml)	69.29±124.45	805.93±890.04	771.16±857.57	<0.001
Blood eosinophils count (%)	2.73±1.93	7.69±4.13	6.88±4.56	<0.001
EASI score	-	28.00±8.01	9.96±5.76	-
IGA scale	-	3.81±0.4	2.16±0.64	-
Pruritus NRS	-	7.16±1.51	2.19±1.01	-
DLQI score	-	17.28±5.36	6.26±3.14	-

Values are presented as mean±standard deviation or number (%). IgE: immunoglobulin E, EASI: Eczema Area and Severity Index, IGA: Investigator's Global Assessment, NRS: Numerical Rating Scale, DLQI: Dermatology Life Quality Index. *Statistical analysis was made between the control and the atopic dermatitis group ($p<0.05$).

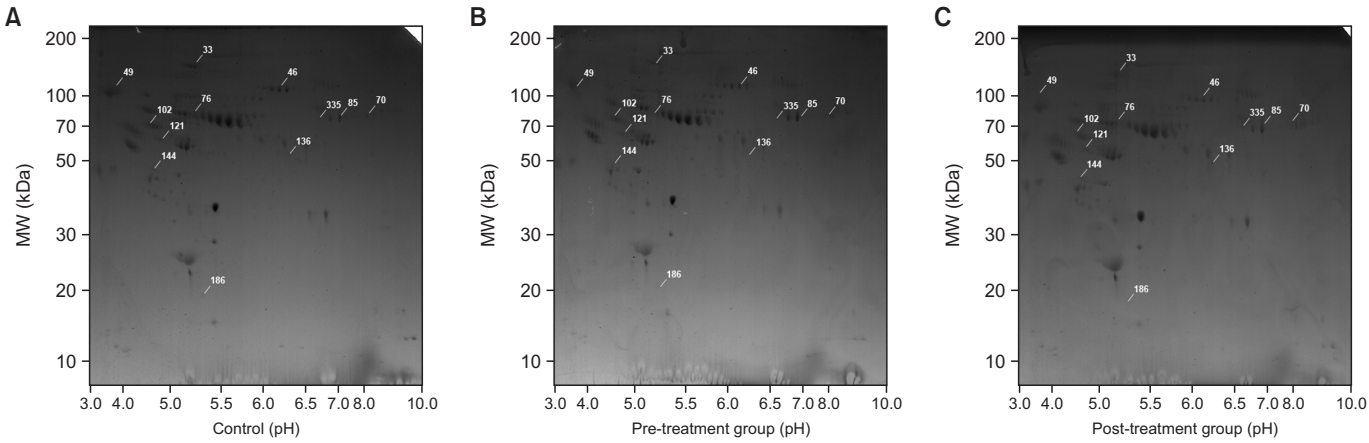

Fig. 1. Two-dimensional gel electrophoresis map of extracted proteins from control (A), pre-treatment (B), and post-treatment group (C) show 12 differentially expressed spots. The spot number corresponded to that in Table 2. MW: molecular weight.

Table 2. Identified protein showing differential expressions in two-dimensional gel electrophoresis image analysis

Group no.	Protein accession number	Protein name	M_r	pI	Sequence coverage (%)	Matches peptide number
33	gi|116117	Ceruloplasmin	122,983	5.44	13	22
46	gi|291922	Complement factor B	86,819	6.55	20	32
49	gi|29535	C1 inhibitor	55,347	6.09	21	37
70	gi|179674	Complement component C4A	194,337	6.65	5	24
76	gi|69990	Alpha-1-B-glycoprotein	52,479	5.65	16	17
85	gi|224983659	Chain A, Complement C3 Beta Chain	71,372	6.82	31	102
102	gi|177890	Prepro alpha-2-thiol proteinase inhibitor	48,936	6.29	15	6
121	gi|28907	Antithrombin	53,025	6.32	15	14
136	gi|28810	Beta-2-glycoprotein	39,598	8.34	2	1
144	gi|190192	Serum paraoxonase	39,895	5.08	14	11
186	gi|116117	Ceruloplasmin	122,983	5.44	1	4
335	gi|40786791	Complement component 3	188,569	6.02	7	30

M_r: molecular mass, pI: isoelectric point.

Validation of serum levels of ceruloplamin

We measured blood Cp levels to validate the results of proteomic analysis. The Cp level was significantly lower in the pre-treatment group (20.87±5.96 mg/dl) and post-treatment group (20.90±5.13 mg/dl) than in the control group (23.56±5.37 mg/dl). However, there was no significant difference between pre and post-treatment group (Fig. 2) in paired t-test.

Relationship between EASI or DLQI score and blood ceruloplamin level

We also analyzed the relationship between various tools of clinical measurement (EASI and DLQI score) and Cp levels in the subgroups of AD patients. There was a significant difference in Cp level between the three severity groups based on EASI and DLQI scores (p<0.01). Specifically, multiple comparisons revealed significant differences between mild and severe groups, and between moderate and severe groups (Fig. 3A). Comparisons between groups according to DLQI score also yielded similar results suggesting significant differences between the group based on DLQI scores of 0~5 and the group with 11 points or higher, and between the group with DLQI score of 6~10 and the group with 11 points or higher (Fig. 3B).

DISCUSSION

AD is one of the most common chronic inflammatory skin diseases. The disease is characterized by itchy dry skin with variable degrees of improving or deteriorating eczematous lesions. It is well known that the clinical features of AD depend on age, race, genetic background, and environmental factors. Also, different treatment methods are indicated depending on the complexity of etiology.

Fortunately, in the last decades, our understanding of diseases with complex etiology has progressed gradually, and facilitated precise target delineation compared with the conventional 'one-size-fits-all' management paradigm[7].

Although many studies have attempted to classify the severity of AD, a reasonable consensus has yet to be established probably due to differences between countries, races, and prevalence rates. In this context, it is very meaningful that a consensus on subjective assessment of severity was published in 2019 after discussions with experts in Korea[8]. Among various instruments utilized for AD severity assessment, the two most commonly used tools for measuring clinical signs of AD include EASI and SCORAD. However, a disadvantage of this subjective assessment of severity is that it requires confidence in intra- or inter-observer reliability and interptretability[9]. Nevertheless, unlike other diseases, these clinical assessment tools play a greater role in patient evaluation than in quantitative and qualitative evaluation of biochemical markers or imaging studies in AD[5].

The definition of the term 'biomarker' may vary, but according to the U.S. Food and Drug Administration (USFDA), it is "a defined characteristic that is measured as an indicator of normal biologic processes, pathologic processes, or responses to an exposure or intervention, including therapeutic interventions". And to elaborate on this in more detail, it is explained as "molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers" (FDA-NIH: Biomarker-Working-Group, 2016). Therefore, reliable biomarkers for patient evaluation can help reduce the observatory differences associated with the use of clinical evaluation tools.

In AD, many biochemical biomarkers have been discovered

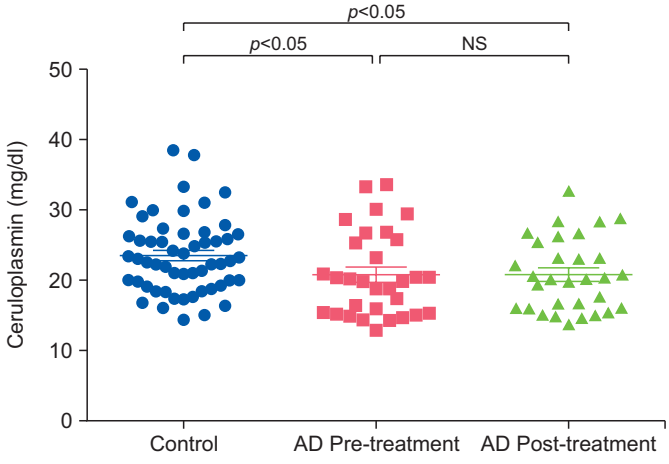

Fig. 2. Comparison of serum ceruloplasmin levels between control, pre-treatment, and post-treatment groups. AD: atopic dermatitis, NS: not significant.

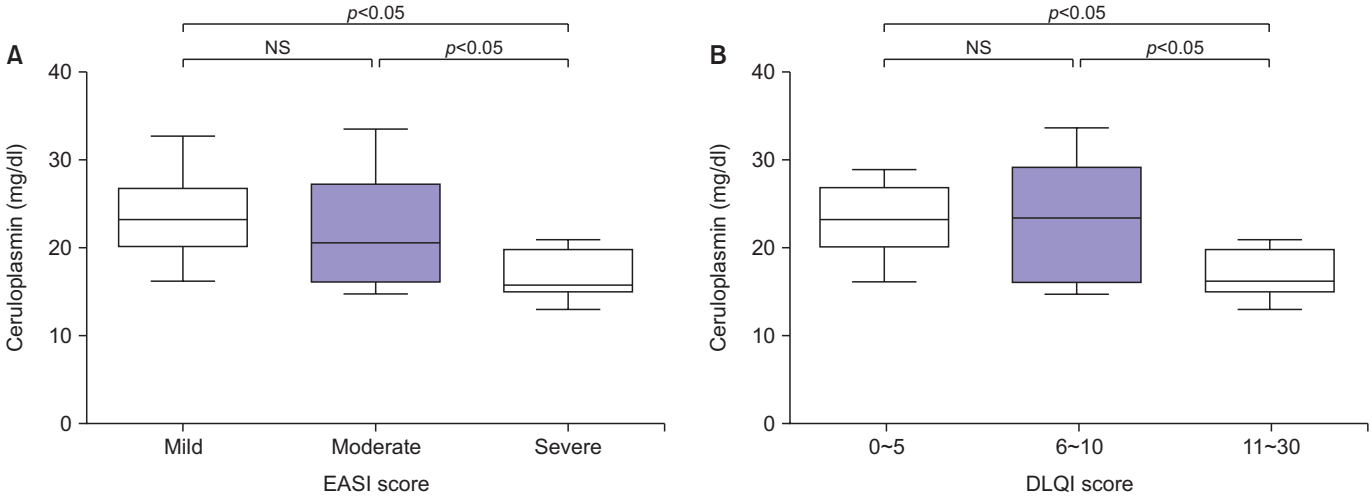

Fig. 3. Comparison of ceruloplasmin levels of three groups based on clinical scores. (A) Eczema area and severity index (EASI); (B) dermatology life quality index (DLQI). NS: not significant.

and studied. Due to pathogenic complexity and the broad clinical spectrum of applications, potential biomarkers of AD may be subdivided based on their suggested use. In particular, the biomarkers are being studied to distinguish disease phenotype, correlate with clinical severity and prognosis, and evaluate of comorbidities.

Although many serum biomarkers related to clinical severity in un-treated AD have been studied, relatively few studies analyzed potential biomarkers related to altered severity before and after treatment, especially systemic treatments such as dupilumab or cyclosporine. However, among them, biomarkers associated with the most reliable treatment response include CCL17/thymus and activation-regulated chemokine (TARC), CCL13/monocyte chemotactic protein (MCP)-4, interleukin (IL)-13, and CCL22/macrophage-derived chemokine (MDC)[10-13].

In this study, we not only attempted to identify new candidate biomarkers but also focused on the effectiveness of the serum biomarker identified following treatment improvement. Thus, we determined the relationship between the patients' pre- and post-treatment clinical scores and candidate biomarkers. We proposed Cp as a potential biomarker of disease severity and clinical improvement in AD, based on comparative proteomics analysis and identification and validation through measurement of serum Cp levels.

Cp is a protein mainly synthesized in hepatocytes. It circulates in the blood and acts as a copper transporter by coupling and transporting most of the serum copper[14]. In addition to metabolism of copper and iron, Cp is involved in coagulation, angiogenesis, defense against oxidative stress, and iron homeostasis[15]. Recent studies reported that Cp is also associated with psoriasis, type 2 diabetes, coronary heart disease, and obesity[16-19]. Although the underlying mechanism has yet to be elucidated, hypothetically, it is likely that Cp mediates the pathogenic effects via apoptosis, cytotoxicity, cell replication, increased oxidative stress and pathogenic gene activation[20].

The role of Cp in AD has yet to be reported. In the present study, we used MS-based proteomics to identify the potential biomarker. Proteomics is one of the most advanced methods for analysis of serum biomarkers. While 2-DE facilitated comparative analysis of protein expression in the 1990s, the subsequent MS analysis allowed accurate measurement of mass and fragmentation spectra of peptides derived from sequence-specific digestion of proteins[21].

After identifying peptide sequences present in a protein sequence database, we listed the proteins with differential expression between the groups. Excluding proteins related to the complement pathway or coagulation factors, Cp was interestingly selected as a candidate for further validation.

Since Cp can be measured without difficulty via a blood test clinically, we validated the results in larger number of patients with AD and normal groups. We conducted a validation study first by comparing the Cp levels in the AD and the control groups, and then investigate the correlation with the clinical indices in the group of patients with AD.

Comparing the Cp levels between control and patient group, adjusted for age and sex, it was confirmed that the Cp level was significantly lower in the AD group. However, no significant difference was found between the groups before and after treatment, as the Cp level did not increase as shown in the proteomic analysis. We hypothesized that because the time interval before and after treatment was only about four months, it would be a rather short period for the change in Cp level in the blood test to be reflected in the post-treatment group, unlike proteomic analysis.

We determined the differences in Cp level according to disease severity. To this end, we investigated the possible relationship between Cp level and clinical severity using EASI, p-NRS, and DLQI, which are the most commonly used clinical measurement tools in practice. Interestingly, when analyzing the relationship between EASI and Cp level, significant differences were found between mild and moderate groups, and mild and severe groups. These results were identical to the result of analysis based on DLQI.

The role of Cp in various allergic diseases including AD has yet to be established. However, in one study investigating the therapeutic relevance of Cp in eosinophilic asthma, the authors reported that serum Cp levels were elevated after treatment with benralizumab suggesting that the rising level of serum Cp may indicate high cellular stress with greater ferroxidase activity in response to treatment[22]. An alternative hypothesis was proposed suggesting that the increased level of Cp may indicate reduction in asthma-induced oxidative stress mediated by anti-inflammatory biologic treatment[23]. Different studies have investigated the effect of Cp therapy in combination with conventional treatments in asthma, and reported a reduction in reactive oxygen species generation and enhanced therapeutic effectiveness[24,25]. Although they are not a study conducted on AD, however, the disease and asthma belong to

the spectrum of atopic disorder, so it has several clinical implications and is worth studying by applying the research results.

This study has some limitations. First, we did not test a large number of patients and controls for validation of candidate protein. Second, although we adjusted for gender or age and excluded subjects with underlying diseases that affected copper or iron metabolism in this study, because the change in Cp level is not specific to AD, further control for other possible variables may be necessary. Third, although similar concerns have been raised in other studies, it was difficult to arrive at a definitive conclusion regarding the changes in Cp preceding or following occurrence or exacerbation of AD. Therefore, longitudinal prospective observational and interventional studies may be needed to further elucidate the relationship between Cp and AD. In the follow-up study, we plan to analyze changes in Cp in AD based on long-term clinical data lasting more than one year. In particular, it is essential to analyze the data of the long-term treatment group in order to clearly observe the decrease in serum Cp level, which could not be confirmed in the short-term treatment group. In addition, in order to establish Cp as a new novel biomarker, another validation study based on immunohistochemical analysis of patients' lesional and non-lesional skin is envisaged.

In conclusion, in this study, we discovered Cp as a potential biomarker reflecting disease severity of AD based on 2-DE and LC-MS/MS analysis, and validated the result by measuring serum Cp level in case-control study. Additionally, the association with clinical severity was statistically established within the patient group.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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