



Contents lists available at ScienceDirect

Allergology International

journal homepage: <http://www.elsevier.com/locate/alit>

Letter to the Editor

Plasma miR223 is a possible biomarker for diagnosing patients with severe atopic dermatitis

Dear Editor,

MicroRNAs (miRNA) are small non-coding RNAs built of 19–25 nucleotides, which bind to specific mRNAs to regulate translation or accelerate mRNA degradation.¹ Recently some reported that there exist the close relationships between miRNAs and several allergic diseases such as asthma, allergic rhinitis, and atopic dermatitis (AD).^{2,3} In addition, miRNAs have been identified not only in cells but also in body fluids such as plasma, serum, and urine. It is thought that miRNAs in body fluids are very possible to be used as non-invasive biomarkers in detection and condition of diseases.

AD is a common chronic inflammatory skin disorder characterized by intense pruritus and recurrent eczematous lesions. It has been reported that some of the miRNAs are overexpressed in skin lesions, serum, and urine of AD patients and are involved in the pathogenesis of AD. It was identified that the miR-223 expressions in AD patients' skin³ and whole blood cells⁴ were upregulated. However, there was no data about the miR-223 expressions of AD patients in body fluid and the relationship between the miR-223 levels and clinical data. In this study, we examined the miR-223 expressions in AD patients' plasma, and the relationship between plasma miR-223 levels and the severity of AD or laboratory data.

A total of 21 adult patients (10 males, 11 females; median age: 43 years old; range: 22–62 years old) with AD were randomly selected and registered for this study. The patients were treated with conventional therapies, including topical corticosteroids and calcineurin inhibitors and systemic antihistamines. Patients that were taking systemic corticosteroids or immunosuppressive agents were not included in this study.

The severity of their AD was estimated using the eczema area and severity index (EASI) score (median score: 17.5; range: 0.4–50.5). Based on the EASI score, the 21 AD patients were divided into 3 groups: the mild (score: <10), moderate (score: 10–20), and severe (score: >20) groups (Supplementary Table 1, 2). The 10 urticarial patients (2 males, 8 females; median age: 38.5 years old; range: 23–61 years old) were randomly selected and registered for this study. The control group, 13 healthy volunteers (5 males, 8 females; median age: 36 years old; range: 22–54 years old) had not taken any medication for at least 2 weeks. All of the subjects provided written consent, and the study protocol was approved by the university ethics

committee and was conducted in accordance with the Declaration of Helsinki. Detailed description of methods is provided in the [Supplementary Material](#) online.

There was a significant difference regarding plasma miR223 expression levels among mild, moderate, and severe AD patients, urticarial patients, and healthy volunteers ($P < 0.01$). The severe AD patients' plasma miR223 expression levels were significantly higher than those of mild AD patients ($P < 0.05$) and those of urticarial patients or healthy volunteers ($P < 0.01$) (Fig. 1A). The experiment using an independent replication dataset showed that plasma miR223 expression levels of severe AD patients were significantly elevated compared with those of urticarial patients or healthy volunteers ($P < 0.05$) (Supplementary Fig. 1). The receiver operating characteristic (ROC) curve establishing cut-off levels between patients with non-severe and severe AD for plasma miR223 expression levels is shown in Figure 1B. The area under the ROC curve was determined to be 0.837 (95% CI: 0.614, 1.00). This procedure was used to obtain a cut-off value, 4.85, giving the maximum efficiency in prediction of non-severe to severe AD with a sensitivity of 86% and a specificity of 93%. The apparent area under the ROC for the selected cut-off value of 4.85 was 0.893, and the optimism-corrected area under the ROC was 0.845. The relative frequency for the cut-off value of 4.85 was 0.65 (Supplementary Table 3).

The correlations between the plasma miR223 expression levels and laboratory data were evaluated. The results showed that miR223 expression levels were significantly correlated with the TARC ($r = 0.54$, $P = 0.042$), but not with the eosinophil counts ($r = 0.30$, $P = 0.286$), IgE ($r = 0.31$, $P = 0.239$), or LDH ($r = 0.49$, $P = 0.065$) (Fig. 2). These results indicate that plasma miR223 expression levels were significantly elevated in severe AD patients and were associated with serum levels of TARC, which stands for a suitable biomarker for monitoring AD severity. Therefore, miR223 levels seem to reflect to profound inflammation similar to TARC levels.

It has been reported that miR223 is associated with the development of inflammation and infection, e.g. inflammatory bowel diseases, type 2 diabetes, and influenza or hepatitis B infection, via regulation of granzyme B, inhibitor of nuclear factor kappa-B kinase subunit alpha (IKK- α), Roquin, and signal transducer and activator of transcription 3 (STAT3).⁵

Histamine is one of the important inflammatory mediators in the pathomechanisms of allergic diseases, including AD. In mammals, histamine-N-methyltransferase (HNMT) is the major histamine degradation enzyme.⁶ Jin *et al.* have shown that the expression of HNMT was upregulated in both AD patients and AD

Peer review under responsibility of Japanese Society of Allergology.

<https://doi.org/10.1016/j.alit.2020.07.010>1323–8930/Copyright © 2020, Japanese Society of Allergology. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

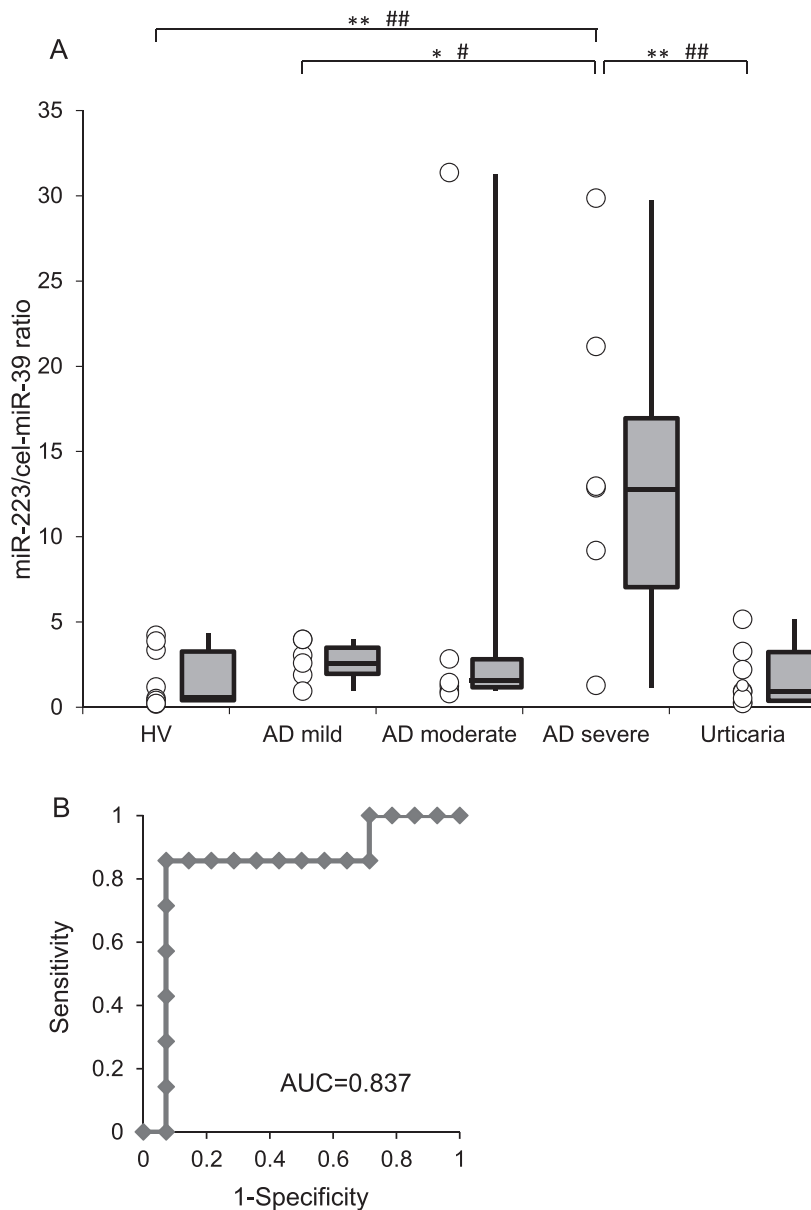


Fig. 1. Plasma miR223 levels of AD patients, urticarial patients and healthy volunteer. **A.** The plasma miR223 expression levels of AD patients ($n = 21$), urticarial patients ($n = 10$), and healthy volunteer (HV) ($n = 13$) were measured. AD patients were divided into 3 groups based on their EASI scores; mild AD (score: <10) ($n = 7$), moderate AD (score: $10-20$) ($n = 7$), and severe AD (score: >20) ($n = 7$). The Tukey–Kramer and Steel–Dwass tests were used to assess the significance of differences. Box plots presenting median, inter-quartile range, and maximum and minimum of the plasma miR223 expression levels are shown for all skin samples. $*P < 0.05$, $**P < 0.01$, Tukey–Kramer and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, Steel–Dwass tests. **B.** The ROC curve establishing cut-off levels between patients with non-severe and severe AD for plasma miR223 expression levels ($n = 21$). AUC, Area under curve.

model mice.⁴ There was one miR223 binding site in the 3'-untranslated region of the HNMT gene.⁴ Therefore, miR223 might be possibly involved in the increase of HNMT to clear away the excessive histamine in AD patients.

Furthermore, Willeit *et al.* have reported that plasma miR223 levels are associated with platelet activation.⁷ In addition to their key functions in hemostasis and thrombosis, platelets are involved in the processes of inflammatory and immune responses.⁸ We have shown that platelets circulate in an activated state in AD patients, and the severity of AD is correlated with the degree of platelet

activation.⁹ In addition, we have demonstrated that platelets play important roles in skin inflammation in AD model mice.¹⁰ These results suggest that miR223 may be related to the pathogenesis of AD through platelet activation.

In conclusion, this study showed that plasma miR223 levels were elevated in severe AD patients, and were related to serum TARC levels. These findings indicate that miR223 is significantly associated with the mechanisms of worsening atopic dermatitis, and indicate that plasma miR223 is a possible biomarker as diagnosis of severe AD.

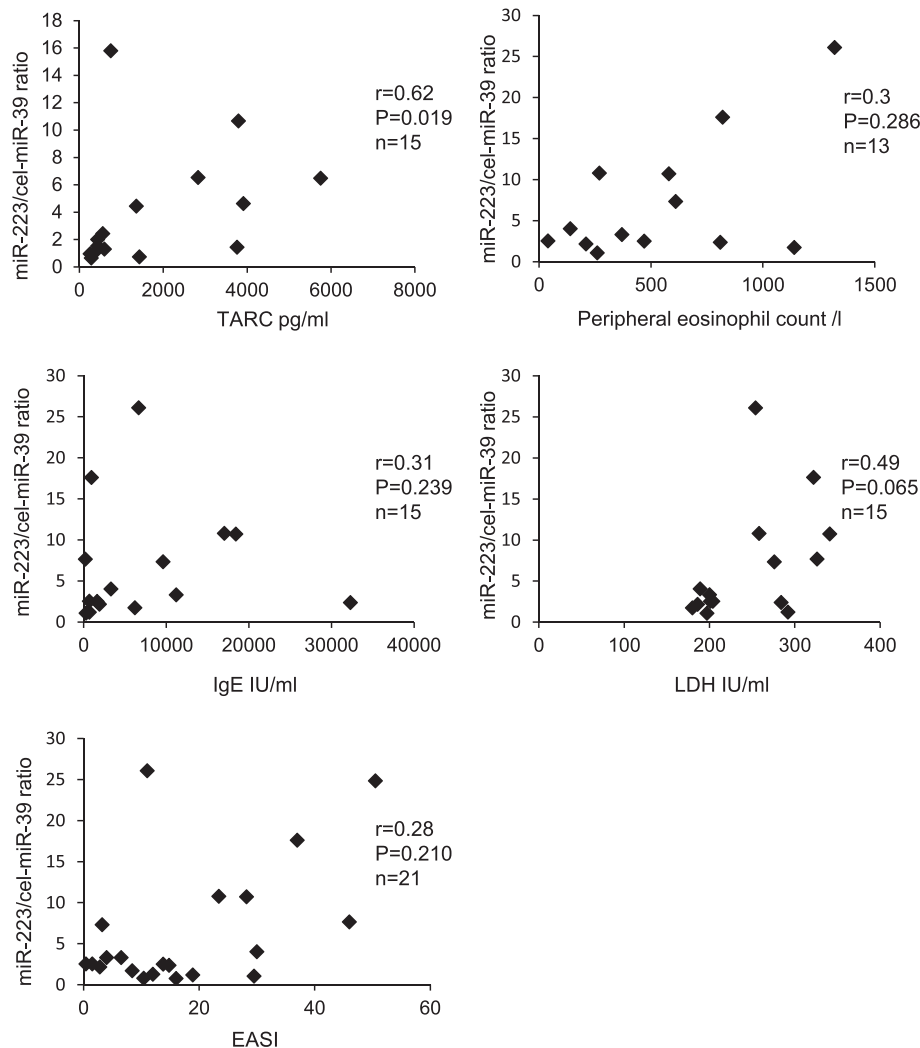


Fig. 2. Correlation between the plasma miR223 level and serum TARC, IgE, LDH, or peripheral eosinophil in AD patients. The correlation between the plasma miR223 level and serum TARC ($n = 15$), IgE ($n = 15$), LDH ($n = 15$), or peripheral eosinophil counts ($n = 13$) was evaluated using Spearman's rank correlation analysis.

Acknowledgements

We thank Hiromi Nishigaki for her technical assistance and Kei Fujikawa for his help with statistical analysis.

This work was supported in part by a grant from Torii Pharmaceutical Co., Ltd. and the Japanese Ministry of Education, Science, Sports, and Culture to R.T.M. and N.K.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2020.07.010>.

Conflict of interest

The authors have no conflict of interest to declare.

Risa Yasuike^a, Risa Tamagawa-Mineoka^{*a}, Naomi Nakamura, Koji Masuda, Norito Katoh

Department of Dermatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

* Corresponding author. Department of Dermatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan.

E-mail address: risat@koto.kpu-m.ac.jp (R. Tamagawa-Mineoka).

References

- Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 2009;**11**: 228–34.
- Dissanayake E, Inoue Y. MicroRNAs in allergic disease. *Curr Allergy Asthma Rep* 2016;**16**:67.
- Rozalski M, Rudnicka L, Samochocki Z. MiRNA in atopic dermatitis. *J Dermatol Sci* 2004;**36**:1–9.
- Jia HZ, Liu SL, Zou YF, Chen XF, Yu L, Yu J, et al. MicroRNA-223 is involved in the pathogenesis of atopic dermatitis by affecting histamine-N-methyltransferase. *Cell Mol Biol* 2018;**64**:103–7.
- Haneklaus M, Gerlic M, O'Neill LA, Masters SL. miR-223: infection, inflammation and cancer. *J Intern Med* 2013;**274**:215–26.
- Arkwright PD. Anti-CD20 or anti-IgE therapy for severe chronic autoimmune urticaria. *J Allergy Clin Immunol* 2009;**123**:510–1.

^a These authors contributed equally to this work.

7. Willeit P, Zampetaki A, Dudek K, Kaudewitz D, King A, Kirkby NS, et al. Circulating microRNAs as novel biomarkers for platelet activation. *Circ Res* 2013;**112**:595–600.
8. Tamagawa-Mineoka R. Important roles of platelets as immune cells in the skin. *J Dermatol Sci* 2015;**77**:93–101.
9. Tamagawa-Mineoka R, Katoh N, Ueda E, Masuda K, Kishimoto S. Elevated platelet activation in patients with atopic dermatitis and psoriasis: increased plasma levels of beta-thromboglobulin and platelet factor 4. *Allergol Int* 2008;**57**:391–6.
10. Tamagawa-Mineoka R, Katoh N, Ueda E, Takenaka H, Kita M, Kishimoto S. The role of platelets in leukocyte recruitment in chronic contact hypersensitivity induced by repeated elicitation. *Am J Pathol* 2007;**170**:2019–29.

Received 28 November 2019
Received in revised form 14 July 2020
Accepted 31 July 2020
Available online xxx

Supplementary Methods

Samples

Peripheral blood (4ml) was obtained from each patient and the healthy volunteers. The blood was transferred into sodium heparin tubes (BD Vacutainer, Franklin Lakes, NJ) and immediately subjected to the three-spin protocol (1500 r.p.m. for 30 min, 3000 r.p.m. for 5 min, and 4500 r.p.m. for 5 min) to prevent contamination by cellular nucleic acids. Plasma was collected and stored at -80°C until further processing.

RNA extraction

Total RNA was extracted from 400 µl of plasma using the mirVana PARIS Kit (Ambion, Austin, TX) and finally eluted into 100 µl of preheated (95°C) Elution Solution according to the manufacturer's protocol.

Quantification of miRNA by qRT-PCR

The amounts of miRNAs were quantified by qRT-PCR using the human TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA). The reverse transcription reaction was carried out with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) in 5 µl of solution containing 1.67 µl of extracted RNA, 0.05 µl of 100mM dNTPs, 0.33 µl of Multiscribe Reverse Transcriptase (50 U/µl), 0.5 µl of 10× Reverse Transcription Buffer, 0.06 µl of RNase inhibitor (20 U/µl), 1 µl of gene-specific primer (has-miRNA-223, Assay ID: 002098; and cel-miR-39, Assay ID: 000200), and 1.39 µl of nuclease-free water. To synthesise cDNA, reaction mixtures were incubated at 16°C for 30 min, at 42°C for 30 min, and at 85°C for 5 min, and then were held at 4°C. Next, 1.33 µl of cDNA was amplified using 10 µl of TaqMan 2× Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems), 1 µl of gene-specific primers/probe, and 7.67 µl of nuclease-free water in a final volume of 20 µl. Quantitative PCR was run on a StepOnePlus PCR system (Applied Biosystems), and reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Cycle threshold (Ct) values were calculated with the StepOne Software (Applied Biosystems).

As previously reported,¹ we used an approach for data normalization based upon spiking the sample with synthetic RNA oligonucleotide, cel-miR-39, which does not exist in the human genome. *C. elegans* cel-miR-39 was purchased as a custom-made RNA oligonucleotide (Qiagen, Valencia, CA). The oligo used for spiking, as a mixture of 25 nmol of oligonucleotide in 5 µl of total volume of water, was introduced after the addition of 2× Denaturing Solution (Ambion) to the plasma sample to avoid degradation by endogenous plasma RNases. As a control for each RNA sample, cel-miR-39 was used for TaqMan qRT-PCR assays (Applied Biosystems) as described earlier. We normalized the data across samples using the $2^{-\Delta\Delta C_t}$ method relative to cel-miR-39.

Statistical analysis

All data are given as median and range. Statistical significance was assessed using one-way analysis of variance followed by the Tukey-Kramer or Steel-Dwass test. The receiver operating characteristic curve was prepared and analyzed using data from patients with AD. For each ROC curve, the Youden Index was calculated. For a selected cut-off value by Youden's index, we estimated the optimism-corrected area under the ROC using bootstrap method (resampling 1000 times) as the internal validation.² The relative frequencies for the cut-off values selected by Youden's index on the bootstrap samples were also calculated. Spearman's rank correlation analysis was used to evaluate the relationships between the miR223 expression and TARC, IgE, LDH, or eosinophil counts. *P*-values of less than 0.05 were considered to be significant in all analyses.

Supplementary references

S1. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection, *PNAS* 2008; **105**: 10513-10518.

S2. Harrell F. Regression Modeling Strategies; With Applications to Linear Models, Logistic and Ordinal Regression, and Survival Analysis. Germany: Springer; 2015.

Supplementary Table 1. Characterization of the subjects whose data are shown in Figure 1.

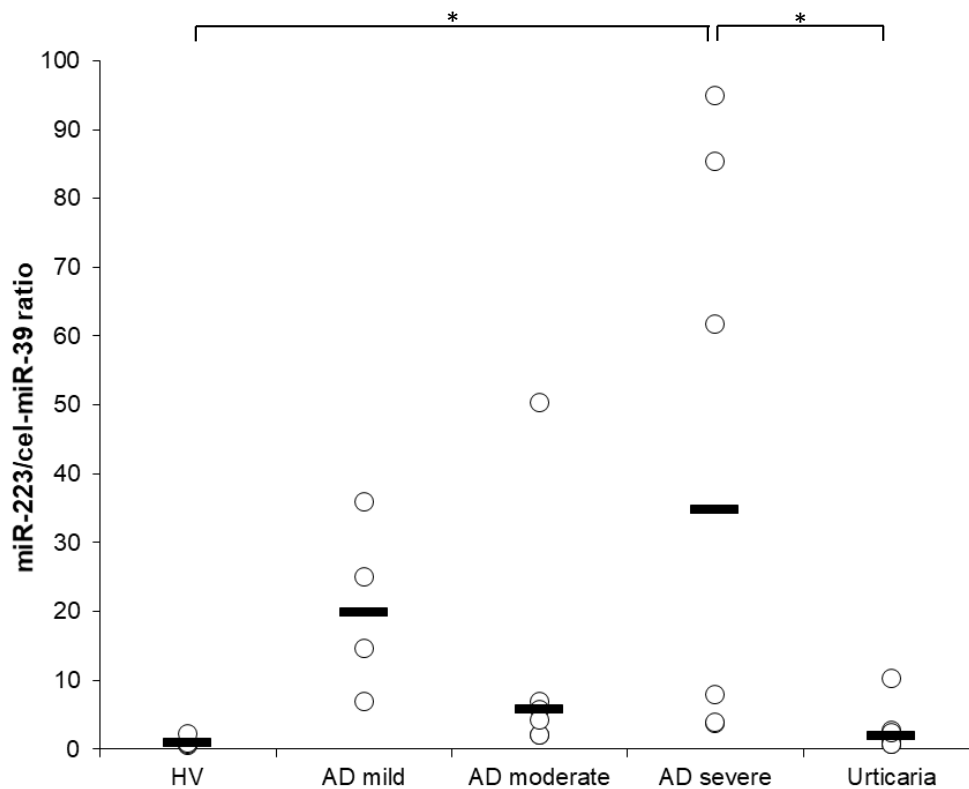
	AD patients		Urticarial patients	Healthy subjects
Age (years)	Median: 43; range: 22-62		Median: 38.5; 23-61	Median: 36; 22-54
Number	21		10	13
AD Severity	Mild	7	—	—
	Moderate	7		
	Severe	7		
Peripheral eosinophil count	Median: 470/ μ l; range: 40-1320/ μ l; reference range 50-590/ μ l		—	—
Serum IgE levels	Median: 3279 IU/ml; range: 201-32284 IU/ml; reference range 0-380 IU/ml		—	—
Serum LDH levels	Median: 254 IU/l; range: 180-341 IU/l; reference range 140-243 IU/l		—	—
Serum TARC levels	Median: 751 pg/ml; range: 255-5754 pg/ml; reference range: 0-499 pg/ml		—	—

Supplementary Table 2. Characterization of the subjects whose data are shown in Supplementary Figure 1.

	AD patients		Urticarial patients	Healthy subjects
Age (years)	Median: 42; range: 22-53		Median: 45; 24-61	Median: 24; 24
Number	17		9	4
AD Severity	Mild	4	—	—
	Moderate	7		
	Severe	6		
Peripheral eosinophil count	Median: 540/ μ l; range: 140-1320/ μ l; reference range 50-590/ μ l		—	—
Serum IgE levels	Median: 4728 IU/ml; range: 201-32284 IU/ml; reference range 0-380 IU/ml		—	—
Serum LDH levels	Median: 256 IU/l; range: 180-326 IU/l; reference range 140-243 IU/l		—	—
Serum TARC levels	Median: 1055 pg/ml; range: 287-3913 pg/ml; reference range: 0-499 pg/ml		—	—

Supplementary Table 3. The relative frequency for the cut-off value of 4.85

Cut-off value	1.29	4.85	9.20	12.87	12.97	21.16	Total
Relative frequency	0.01	0.65	0.24	0.07	0.02	0.01	1



Supplementary Figure 1. Plasma miR223 levels of AD patients, urticarial patients and healthy volunteer.

The plasma miR223 expression levels of AD patients (n = 17), urticarial patients (n = 9), and healthy volunteer (HV) (n = 4) were measured. AD patients were divided into 3 groups based on their EASI scores; mild AD (score: <10) (n = 4), moderate AD (score: 10-20) (n = 7), and severe AD (score: >20) (n = 6). Bars presenting median are shown for the samples. Statistical significance was assessed using one-way analysis of variance followed by the Tukey-Kramer. * $P < 0.05$.