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Original Research

MicroRNA-223 is involved in the pathogenesis of atopic dermatitis by affecting histamine-N-methyltransferase

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Abstract: Atopic dermatitis (AD) is one of the most prevalent skin diseases around the world. Excessive histamine plays a critical role as an inflammatory factor in the pathogenesis of AD. Deregulated microRNAs (miRNAs) were involved in atopic dermatitis by targeting various genes. MiR-223 had been reported to play a vital role in hematopoiesis. In this study, we identified upregulated miR-223 in the whole blood cells of a large group of AD patients. What's more, we found for the first time that one of the major histamine degradation enzymes, histamine-N-methyltransferase (HNMT), was increased in AD patients and AD model mice. Although there was one miR-223 binding site in the 3'- untranslated region of the HNMT gene, HNMT were not inhibited by miR-223. Taken together, it suggested that miR-223 participates in AD through upregulating HNMT indirectly to degrade the excessive histamine.

Key words: Atopic dermatitis; Histamine-N-methyltransferase; Micro RNAs; miR-223.

Introduction

Atopic dermatitis (AD) is the most common skin disease in the world, which affects about 20 % of children and 3 % of adults (1). It has been showed that histamine, the important inflammatory mediator, was increased in the skin and the blood of patients with atopic dermatitis (2). The increase of histamine resulted in accumulation of cellular infiltrate and dysregulated expression of multiple epidermal barrier genes (2). In mammals, histamine-N-methyltransferase (HNMT) is one of the most important enzyme that degrades histamine (3). Excessive histamine in AD patients can be inactivated by HNMT (4). MicroRNAs (miRNAs) are short ssRNA molecules of 19 to 25 nucleotides in length, which can bind complementary sites in the 3'-untranslated region (3'-UTR) of target gene. MiRNAs suppress gene expression by directing translational repression, mRNA destabilization, or a combination of both mechanisms (5). MiRNAs have been studied in a variety of biological functions including organ development, stress response, tumorigenesis, cardiac hypertrophy, immune response and so on (6). Moreover, miRNAs have been involved in allergic inflammation. Some preclinical studies have demonstrated the possible clinical utility of miRNAs for patients with allergic diseases. Moreover, miRNAs may play a role as biomarkers and therapeutic targets in those diseases (7).

Especially, miR-223 has been reported to be involved in inflammation, infection and cancer development through regulating Granzyme B, Inhibitor of nuclear factor kappa-B kinase subunit alphaI (KKa), Signal transducer and activator of transcription 3 (STAT3), CCAAT/enhancer-binding protein beta (C/EBPb), Forkhead box protein O1(FOXO1) and Nuclear factor 1 A-type (NFIA). What's more, miR-223 plays a prominent part in the hematopoietic cell differentiation (8).

A previous study mentioned that miR-223 was increased in the lesional skin from patients with AD, but the mechanism of miR-223 in AD pathogenesis is still unknown (9). In this study, it was found that both miR-223 and its predicted target HNMT were upregulated in the whole blood cells of AD patients. It revealed that miR-223 is involved in AD through indirectly regulation of HNMT expression.

Materials and Methods

Cell culture and transfection

HEK 293T and Human liver cancer cell line HepG2 were purchased from the Typical Cell Culture Collection Committee of the Chinese Academy of Sciences. Both cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplied with 10% Fetal Bovine Se-

rum (FBS) (Gibco, USA) and 1% penicillin/streptomycin. HepG2 cells and HEK 293T cells were transfected with Lipofectamine 2000 according to the instructions.

Patients and healthy donors

Patients with AD (83 male/88 female, mean age: 12.15 ± 11.21) were recruited from the Department of Dermatology, Peking University Shenzhen Hospital. Atopic dermatitis diagnoses were determined with the Hanifin and Rajkagn diagnostic criteria. Healthy donors (79 male/ 92 female, mean age: 12.95 ± 11.09) were also recruited from Peking University Shenzhen Hospital. This study was approved by the human ethics committee of the Peking University Shenzhen Hospital, and written informed consent was obtained from all subjects.

Mice and AD model

C57/ B6 mice were obtained from the Experimental Animal Center in Guangdong. C57/ B6 mice were epicutaneously sensitized twice and challenged with OVA as previous description(10). In brief , OVA solution (100 μg OVA / 100 μl PBS) was placed on a patch of 1 cm^2 filter paper , pasted to the depilated skin and fixed with gaze and bandage for 1 week. All of the mice had three-1-week exposure separated by 2-week break from each other. At the beginning, mice (6–8 per group) were between the ages of 8 and 9 weeks. Mice were killed at the end of the third challenge. For MC903 treatment as previous description, MC903 (Sigma, USA) dissolved in ethanol was topically applied on each ear (1.125nmol MC903 / 25 μL) of 6- to 8-week-old female C57/B6 mice every day. 25μL ethanol was applied on ear of mice as vehicle control (11). All procedures were approved by the Animal Use and Care Committee of Peking University Shenzhen Hospital.

RNA extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from blood and HepG2 cells with TRIzol (Invitrogen, USA) according to the manufacture. RNA quantity was measured by Nanodrop 2000 (Thermo Fisher Scientific).Total RNA (1μg) was reverse transcribed by Reverse Transcription Kit (Promega, USA) following the instructions. 1μl cDNA was amplified by qRT-PCR with CFX-96 Real Time PCR Instrument (Bio-Rad). GAPDH was used as the internal control. All the primers used in this study are listed in Table 1.

Western Blot

Protein samples were lysed in RIPA buffer with Cocktail (Sangon), quantified with BCA protein assay (Transgene) and separated with 12% SDS-PAGE gel. The separated proteins were transferred to PVDF membrane and then incubated with primary antibodies at 4 °C overnight and secondary antibodies for 1h at room temperature. Films were developed with the Western Lightning PLUS (NEL105001EA, PerkinElmer).

Dual luciferase reporter assay

The 3' untranslated region (3'UTR) of HNMT gene was cloned into the psiCHECK-2 luciferase vector (Promega, USA). The primmer used was listed in Table 1. HEK 293T cells were transfected with reporter vectors, miR-223 mimic and mimic control (or reporter

Table 1. List of primers used in this study.

Primers	Sequences(5'-3')
Reporter Assay	
HNMT-F	CCGCTCGAG CATTAATGTA GATAAAGCAC
HNMT-R	TATAGCGGCCGCTTACCATATGCAAGGCACC
qRT-PCR	
MiR-223-F	CCGCTCGAGGAGCTTCCAGCTGAGCACTGGG
MiR-223-R	CGACGCGTTATTGCGCCCCCATCAGCACT
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
HNMT-F1	ACCAAAGTAGAATGTTGGAG
HNMT-R1	TTGGTACCTAAGAGACTATG
GAPDH-F	GCACCGTCAAGGCTGAGAAC
GAPDH-R	TGGTGAAGACGCCAGTGGA
mHNMT-F	CACATCTTCTGATCTTGCGCA
mHNMT-R	TCTGCTTTGAGATCCAGTGGT
mGAPDH-F	AACTTTGGCATTGTGGAAGG
mGAPDH-R	GGATGCAGGGATGATGTTCT
c-Jun-F	GCGGAGACAGACCAACTAGA
c-Jun-R	GCGGAGACAGACCAACTAGA
c-Fos-F	AAGACCGAGCCCTTTGATGA
c-Fos-R	GGAAGACGTGTAAGCAGTGC
NFE2L1-F	AAAACATCTGCACACCCACG
NFE2L1-R	TGTGCGCAAATTGAAGTCCA
MALT1-F	AACCCAGAGTCAAAGGCAGT
MALT1-R	AACATCCAGCTGTGACCAC

vectors, miR-223 inhibitor and inhibitor control) (RIBOBIO, China) in 24-well plate. After 24 hours, luciferase activities were measured with the dual-luciferase assay system (Promega, USA) according to the manufacturer's instructions. The experiments were performed in triplicates.

Results

MiR-223 is upregulated in AD patients and OVA induced animal model

It was found that miR-223 was significantly upregulated in blood from atopic dermatitis patients (n=171) in comparison with that from healthy donors (n=171) (Fig 1a), which was consistent with previous study of lesion skins from AD patients. As shown in the Fig. 1b, the area under curve (AUC) of Receiver-operating characteristic (ROC) is 0.993, estimated with 95% confidence interval (P<0.0001), suggesting the reliable diagnostic accuracy of miR-223. Thus miR-223 in the whole blood cells might be a biomarker for diagnosing atopic dermatitis.

To confirm high expression of miR-223 in AD patients, the OVA induced AD model in C57BL/6J mice was established. The haematoxylin and eosin (HE) staining of mouse skin tissue showed obvious epidermal hyperplasia, para-aceratosis, and eosinophils infiltration in OVA induced animal model (Fig.S1). Consistently in patients with AD, the expression of miR-223 in the whole blood cells of OVA induced mice model was significantly higher than that of the control group (Fig 1c). Another mouse model for atopic dermatitis with topical application of MC903 (a Vitamin D3 analog) nicely recapitulates important hallmarks of lesional AD (11,12). So this efficient and less time-consuming way we introduced to test the trend of miR-223 level and the severity of lesional AD. As Fig.S2 shown, higher level of miR-223 accompanied more severe AD lesions with the increase of MC903 treatment duration.

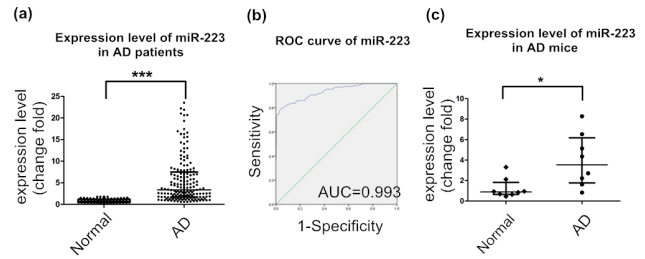

Figure 1. MiR-223 is highly expressed in blood cells of atopic dermatitis patients and OVA induced AD mice. (a) qRT-PCR analysis of miR-223 in atopic dermatitis and healthy blood cells. (b) Receiver-operating characteristic (ROC) curve of miR-223. AUC value was presented by the estimate with 95% confidence interval. (c) qRT-PCR analysis of miR-223 in OVA induced AD mice and control mice. U6 was used as an internal control. ***P < 0.001. The normalized expression data was analyzed using $2^{-\Delta\Delta Ct}$ method with students' T-test.

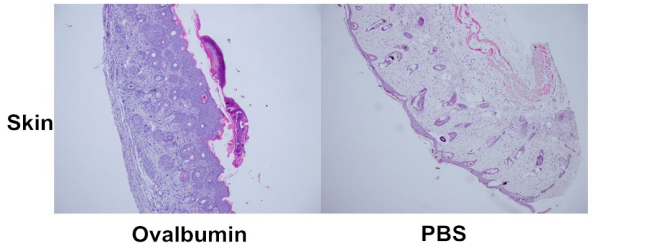

Figure S1. OVA-treated mouse skin showed obvious AD-like skin inflammation. The lesion skins were observed by the hematoxylin and eosin staining. OVA-treated skin (left) showed epidermal hyperplasia, para-aceratosis and eosinophils infiltration, while PBS-treated skin (right) showed no obvious changes.

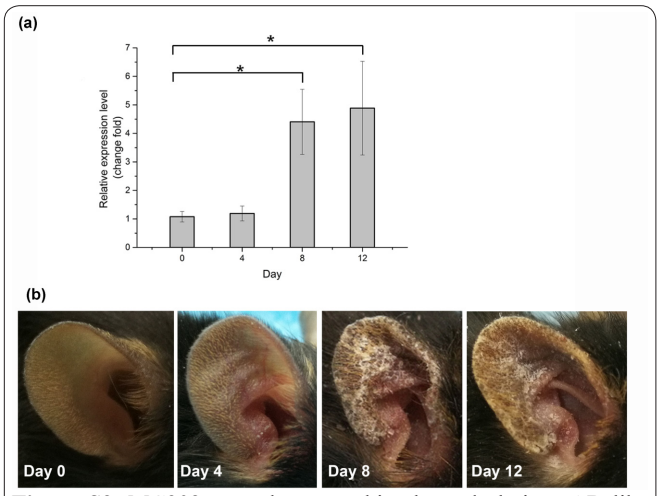

Figure S2. MC903-treated mouse skin showed obvious AD-like skin inflammation. (a) qRT-PCR analysis of miR-223 in AD mice with the increase of MC903 treatment duration. U6 was used as an internal control. *P < 0.05. The normalized expression data was analyzed using $2^{-\Delta\Delta Ct}$ method with one-way ANOVA. (b) More severe AD lesions with the increase of MC903 treatment duration.

The expression of HNMT was upregulated by miR-223 indirectly

To better understand the role of miR-223 in atopic dermatitis, bioinformatic analysis (including miRanda and TargetScan algorithm) were performed. HNMT turned out to be one of the putative targets. The miR-223 binding sequence present at the 3'-UTR of HNMT was cloned downstream of the firefly luciferase reporter gene and then cotransfected with miR-223 mimic or negative control into HEK 293T cells. The luciferase activity of the constructed reporter was not significantly changed when miR-223 mimic was co-transfected (Fig.2a). Moreover, transfecting miR-223 mimic in HepG2 cells caused the increased protein level of HNMT (Fig.2b). However, no obvious change of HNMT has been found in the luciferase assay and western blot experiment with MIR-223 knocked down (Fig.S3).

HNMT was increased in both AD patients and the OVA induced AD model.

To verify the results in the miR-223 regulation of

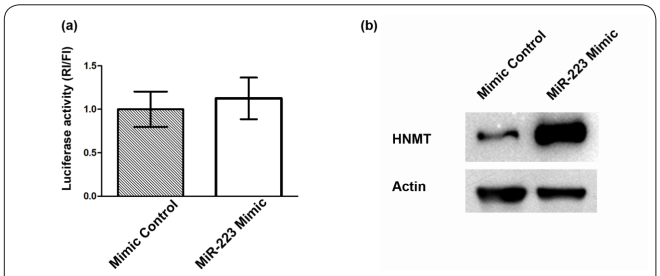

Figure 2. HNMT was upregulated by miR-223. (a) Target gene was verified by luciferase reporter assay. Data are represented as mean ± SD. (b) The protein level of HNMT in HepG2 cells transfected with miR-223 mimic was determined by Western Blotting.

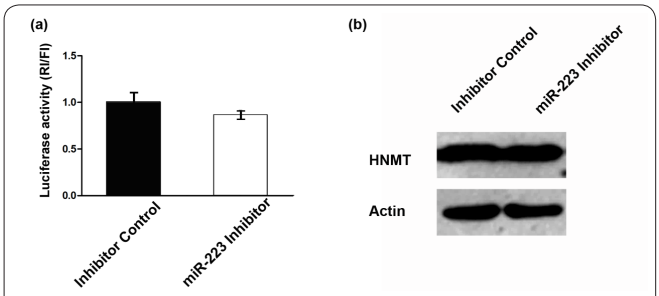

Figure S3. The expression level of HNMT was not changed by miR-223 inhibitor. (a) Target gene was verified by luciferase reporter assay. Data are represented as mean ± SD. (b) The protein level of HNMT in HepG2 cells transfected with miR-223 inhibitor was determined by Western Blotting.

HNMT, we detected the mRNA level of HNMT in the whole blood cells of a large group of AD patients, in which the miR-223 was upregulated. As shown, HNMT also turned out to be significantly increased in AD patients (Fig.3a). Furthermore, we detected the HNMT expression level in the whole blood cells of OVA induced AD mice. As in the case of AD patients, HNMT was obviously increased in the AD group compared with the mice in the control group (Fig.3b).

MiR-223 overexpression caused c-Jun dysexpression.

Since HNMT was not directly targeted by miR-223, other mechanisms must be explored. Several transcription factors containing binding sites in the HNMT promoter region were predicted. We detected the expression level of these transcription factors (including c-Jun, c-Fos, nuclear factor erythroid-2-like 1 (NFE2L1), lymphoma translocation protein 1(MALT1)). As shown in Fig.4, c-Jun is increased after miR-223 overexpression in HepG2 cells, while others were not obviously changed (Fig.4).

Discussion

Our study verified the upregulation of miR-223 in blood from a large atopic dermatitis patient population and the OVA induced AD model in C57/B6 mice. MiR-223 has been reported to be aberrant expressed in various infection and inflammation diseases, such as sepsis, influenza or hepatitis B infection, type 2 diabetes,

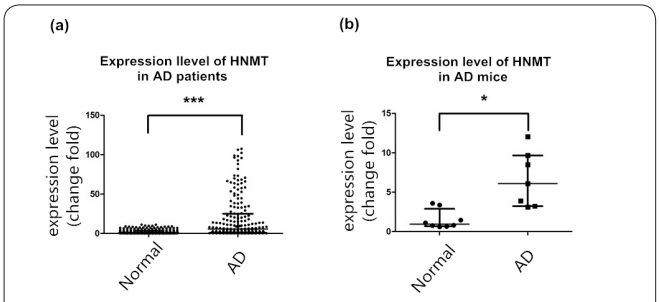

Figure 3. HNMT was increased in AD patients and OVA induced AD mice. (a) The mRNA level of HNMT in AD patients and healthy control was determined by qRT-PCR. (b) The mRNA level of HNMT in OVA induced AD mice and corresponding control mice was determined by qRT-PCR. GAPDH was used as an internal control. ***P < 0.001. The normalized expression data was analyzed using $2^{-\Delta\Delta Ct}$ method with students' T-test.

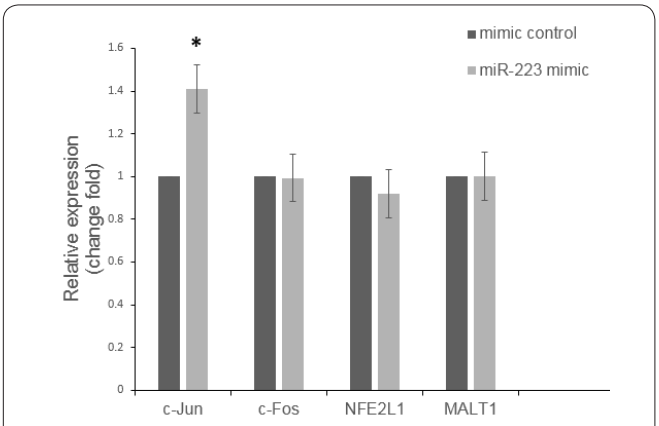

Figure 4. Overexpression of miR-223 affected expression of c-Jun. The mRNA level of c-Jun, c-Fos, NFE2L1, and MALT1 in HepG2 cells transfected with miR-223 mimic and mimic control was determined by qRT-PCR. GAPDH was used as an internal control. *P < 0.05. The normalized expression data was analyzed using $2^{-\Delta\Delta Ct}$ method with students' T-test.

rheumatoid arthritis, inflammatory bowel disease (8). Particularly, miR-223 plays a crucial role in the differentiation of granulocyte, T cells and eosinophil (13). It is especially notable that the upregulated miR-223 exerts its function by targeting IGFR1 and influencing eosinophil development in allergic diseases, such as asthma, eosinophil esophagitis and atopic dermatitis (7,14).

Though both algorithms showed HNMT was one of miR-223 targets, HNMT mRNA was not changed while protein was increased in the miR-223 overexpressed cells. We tried to find whether transcript factors regulating HNMT expression are targets of miR-223. However, the expression of c-Jun was increased in the miR-223 overexpressed cells, which suggested that c-Jun was not a direct target of miR-223.

Accordingly, we firstly identified the significant increasing level of HNMT in the AD patients and AD mice. In the pathogenesis of atopic dermatitis, increased histamine is considered to be associated with pruritus and the broken epidermal barrier (15). As our results shown, increased HNMT is needed to clear away the excessive histamine in subjects with AD. In other studies among the atopic dermatitis patients, it was reported that several common single nucleotide polymorphisms (SNPs) resulted in the decrease of the HNMT activity (16,17). It indicated that high level of HNMT expression induced by miR-223 could make up its deficiencies of activity.

Taken together, for the first time we reported HNMT, the enzyme in histamine metabolism, is increased in the whole blood cells of AD patients. Meanwhile, we revealed that the upregulated miR-223 was involved in the pathogenesis of atopic dermatitis by indirectly promoting HNMT expression to degrade the excessive histamine.

Acknowledgments

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JCYJ20150403091443305). Wei Zhang, Bo Yu and Yi Xiong designed the experiments; Hui-Zhen Jia and Sheng-Lin Liu performed experiment; Hong-Yu Zhang, Jun Wan and Lin Yu analyzed the data; Qi Chen, Yan-Fen Zou and Xiao-Fan Chen wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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