

Increased miR-424-5p expression in peripheral blood mononuclear cells from patients with pemphigus

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Abstract. Pemphigus is an autoimmune disease that causes blisters and erosions in the skin and mucous membranes. The development of pemphigus is associated with the imbalance of T-cell and humoral responses. MicroRNAs (miRNAs) can regulate many cell functions. However, whether miRNA expression is altered in peripheral blood mononuclear cells (PBMCs) during the pathogenesis of pemphigus has not been clarified. The aim of the present study was to examine the miRNA expression profiles of PBMCs from patients with pemphigus. The expression profiles of miRNAs in PBMCs from patients with active pemphigus (n=3) and healthy subjects (n=3) were analyzed by microarray. The relative levels of miR-424-5p expression in PBMCs from 9 patients and controls were validated by RT-qPCR. The functional and biological processes of the differentially expressed miRNAs were analyzed by bioinformatics. There were 124 differentially expressed miRNAs in PBMCs from the patients with pemphigus, compared with healthy controls, including 71 that were upregulated ($P < 0.05$, fold change > 2), and 53 that were downregulated ($P < 0.05$, fold change < 0.5). miR-424-5p was highly expressed in patients with pemphigus. Bioinformatics analysis indicated that the genes targeted by miR-424-5p were involved in intracellular signaling cascades, phosphate metabolism and regulation of kinase activity. The predicted target genes were associated with the T-cell receptor and mitogen-activated protein kinase signaling pathways as well as others. In conclusion, the results have demonstrated the miRNA expression profile, and verified that miR-424-5p was upregulated in PBMCs from patients with pemphigus. The biological function and potential pathways of miR-424-5p in pemphigus were predicted. Thus, miR-424-5p may contribute to the pathogenesis of pemphigus.

Introduction

Pemphigus is a group of blistering autoimmune diseases affecting the skin and mucous membranes (1). Although the cause of pemphigus remains unknown, it is thought that humoral responses to desmogleins are associated with the development of bullation. Desmoglein 1 and 3 are the main components of desmosomes, involved in connecting keratinocytes. Autoantibodies against desmogleins promote the hydrolysis of plasminogen into plasmin in keratinocytes, leading to the loss of cell-cell adhesion and the formation of blisters. It is well known that CD4⁺ T cells can regulate humoral responses and may contribute to the pathogenesis of pemphigus. Pro-inflammatory T cells enhance humoral responses while regulatory T cells (Tregs) inhibit B-cell activation and antibody production (1-4). However, T-cell activation and functional regulation of the humoral response during the pathogenesis of pemphigus have not been clarified.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate growth, development, aging, apoptosis and other important processes *in vivo* (5). Currently, thousands of miRNAs have been identified in humans and many of them are expressed in immune cells. They are involved in regulating maturation, differentiation and signal transduction (6-8). Some miRNAs can regulate the development and progression of autoimmune diseases by modulating T- and B-cell function (9,10). However, to the best of our knowledge, there is no information on how miRNA expression profiles change in human peripheral blood mononuclear cells (PBMCs) during the pathogenesis of pemphigus.

To explore the potential role of miRNAs in the pathogenesis of pemphigus, we characterized the expression profiles of miRNAs in PBMCs from patients with pemphigus and age- and gender-matched healthy subjects by microarray analysis. Furthermore, we validated the high levels of miR-424-5p expression in PBMCs from patients with pemphigus. The potential gene targets of miR-424-5p and their possible functional networks were predicted by bioinformatics.

Patients and methods

Human subjects. Patients with pemphigus diagnosed according to their clinical, histopathological, and immunological parameters were recruited at the Nanfang Hospital of Southern

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Medical University (Guangzhou, China) (11). They had new blisters and had not been treated with immunosuppressive drugs. The exclusion criteria were serious systemic disease, infection, tumors or any other autoimmune disease. The age- and gender-matched healthy subjects were recruited from the Physical Examination Center of the same hospital. Written informed consent was obtained from individual participants and the experimental protocol was approved by the Institute Review Board of Nanfang Hospital of Southern Medical University.

Blood sample collection and pretreatment. A volume of 5 ml of venous blood was obtained from the patients and controls, and plasma samples were prepared. PBMCs were isolated by Ficoll-Hypaque (TBD Science, Tianjin, China) density gradient centrifugation, lysed in Mix RNAiso blood buffer (Takara Bio, Inc., Otsu, Japan) and stored at -80°C until use.

Isolation and quality control of RNA. Total RNA was extracted from individual PBMC samples and purified using the miRNeasy Mini kit (cat. no. 217004; Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The integration of the purified RNA was characterized in the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

miRNA microarray analysis. The total RNAs were dephosphorylated by phosphatase and incubated with the Labeling Spike-In kit (Agilent Technologies Inc.) at 37°C for 30 min. The dephosphorylated RNAs were denatured by dimethyl sulfoxide (DMSO) and subsequently incubated at 16°C in a circulating water-bath or cool block for 2 h. The labeled RNAs were purified with spin columns to remove DMSO in the samples, dried in vacuum concentrators at $45\text{--}55^{\circ}\text{C}$ for 1 h and dissolved in nuclease-free water. The dissolved RNAs were mixed with Hyb Spike-In solution (Agilent Technologies Inc.) to assemble the hybridization mixture. The mixture was hybridized to the Agilent Human miRNA array V19.0 (Agilent Technologies Inc.), which covers 2,006 human miRNAs, at 55°C for 20 h. The arrays were washed with the Gene Expression Wash Buffer kit and subsequently scanned by the Agilent Microarray Scanner (both from Agilent Technologies Inc.). Data on miRNA microarray images were extracted by Feature Extraction software 10.7.1.1 and normalized by Gene Spring software 12.6 (both from Agilent Technologies, Inc.). The similarity between the samples was analyzed by the principal component analysis (PCA) and correlation plot.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was used to validate the expression level of differentially expressed miRNAs in 9 patients and 9 healthy controls. Total RNAs were isolated from PBMCs and subsequently reverse transcribed to cDNAs using miScript II RT kit (Qiagen, Valencia, CA, USA). Briefly, $1\ \mu\text{g}$ total RNA was used as template in the reaction, and reverse transcribed in a $20\text{-}\mu\text{l}$ reaction mixture containing $4\ \mu\text{l}$ 5X miScript HiFlex Buffer, $2\ \mu\text{l}$ 10X miScript Nucleics Mix, $2\ \mu\text{l}$ miScript Reverse Transcriptase Mix and $12\ \mu\text{l}$ H_2O . The thermal profile conditions for reverse transcription were 37°C for 60 min, 95°C for 5 min and 4°C indefinitely. The prepared cDNAs were stored at -20°C until use.

The relative levels of miRNA transcripts normalized to the internal control (U6) were determined by qPCR using the miRcute miRNA qPCR Detection kit containing a universal 3' primer (SYBR-Green; Tiangen Biotech Co., Ltd., Beijing, China) on a 7900 HT Sequence Detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The 5' sequences of primers for U6, 5'-GCTCGCTTCGGCAGCACAT-3' and for miR-424-5p, 5'-CAGCAGCAATTCATGTTTAAAA-3' (Invitrogen Life Technologies). The reaction mixture contained $5\ \mu\text{l}$ 2X miRcute miRNA Premix, $0.8\ \mu\text{l}$ 50X ROX Reference Dye, $0.2\ \mu\text{l}$ forward primer, $0.2\ \mu\text{l}$ reverse primer, $1\ \mu\text{l}$ prepared RT products and $2.8\ \mu\text{l}$ H_2O . All reactions were ran in triplicate at 95°C for 2 min, and subjected to 40 cycles at 94°C for 15 sec and 60°C for 1 min. The data were first normalized to the levels of U6 and analyzed by $2^{-\Delta\Delta\text{C}_q}$.

Statistical analysis. The differences between groups were analyzed using the Student's t-test. Data from the miRNA microarrays were considered differentially expressed when there was a P-value of <0.05 and fold changes were >2 -fold or <0.5 -fold. The target genes of differentially expressed miRNA were predicted with miRanda, miRWalk and TargetScan. The biological processes and signaling pathways of the differentially expressed miRNAs were analyzed by the Gene Ontology (GO) analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Microarray analysis. To analyze the miRNA expression profiles, 3 patients with pemphigus and 3 healthy subjects were recruited and RNA from PBMCs was extracted for microarray analysis. The similarity and correlation of miRNA expression profiles between the patients and healthy subjects were analyzed by the PCA and correlation plot (Fig. 1). There was clear separation in the miRNA profiles between the 2 groups and the intra-group miRNA profiles had high correlation coefficients. Therefore, the samples in patients were different from healthy controls according to the biological characteristics, indicating that the samples were well selected. There were 71 upregulated ($P<0.05$, fold change >2) and 53 down-regulated ($P<0.05$, fold change <0.5) miRNAs on the Agilent Human miRNA array V19.0. Of these, miR-424-5p was one of the miRNAs that was upregulated by $>1,000$ -fold in the patients with pemphigus (Table I).

RT-qPCR analysis of miR-424-5p. To validate the higher expression of miR-424-5p, another 9 patients and age- and gender-matched healthy subjects were recruited and the levels of miR-424-5p in their PBMCs were determined by RT-qPCR. The relative levels of miR-424-5p normalized to U6 in the PBMCs from the patients were significantly higher than in the healthy subjects (Fig. 2).

Bioinformatics analysis. The target genes of miR-424-5p were predicted using miRanda, miRWalk and TargetScan. As predicted, an intersection of 3,539 miRNAs were regulated by miR-424-5p. Of these, 2,430 miRNAs were expressed in

Table I. Ten most upregulated or downregulated miRNAs in PBMC from patients with pemphigus vs. healthy controls.

miRNAs	Fold change	P-value	miRNA	Fold change	P-value
miR-424-5p	1507.524	0.0010	miR-595	0.007866	0.0060
miR-338-3p	1451.776	0.0021	miR-557	0.006176	0.0049
miR-340-5p	1430.471	0.0018	miR-4726-5p	0.005665	0.0062
miR-30e-3p	942.3967	0.0007	miR-4472	0.005267	0.0001
miR-145-5p	908.3626	0.0001	miR-4632-5p	0.004766	0.0004
miR-130b-3p	781.2204	0.0060	miR-5088	0.004145	0.0004
miR-199b-5p	772.3043	0.0230	miR-3648	0.003781	0.0010
miR-128	732.1749	0.0023	miR-4430	0.00352	0.0002
miR-590-5p	722.1934	0.0479	miR-4767	0.003312	0.0040
miR-324-5p	714.0275	0.0010	miR-1180	0.003013	0.0005

MicroRNAs, miRNAs; PBMC, peripheral blood mononuclear cell.

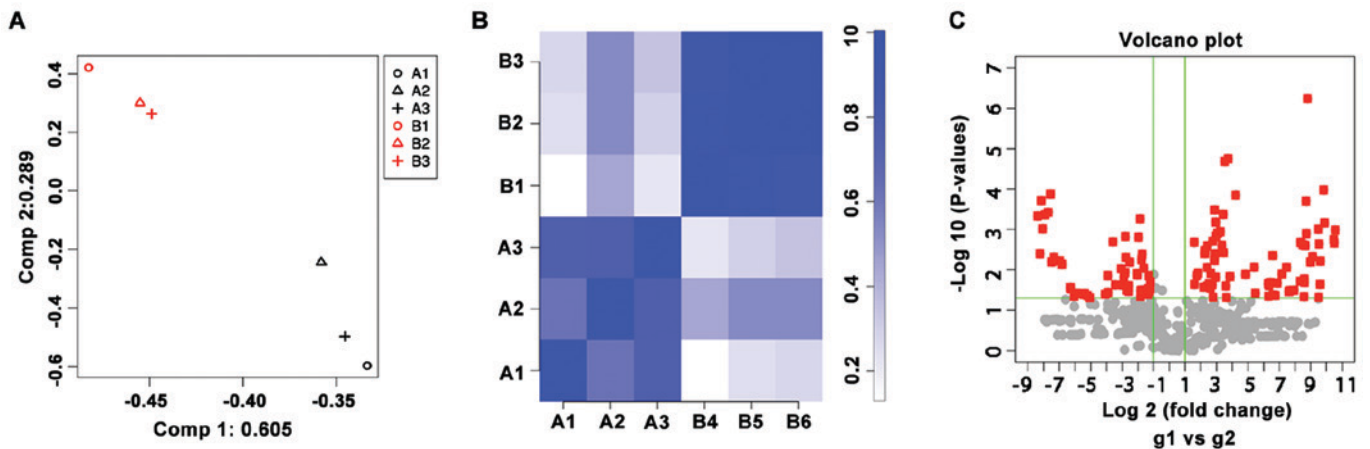


Figure 1. (A) The PCA analysis of miRNA microarray data. Group A represents samples from patients, while group B represents samples from healthy controls. (B) Correlation plot analysis of miRNA microarray data. The correlation coefficients between the samples are expressed by the shades of color. (C) Volcano plot analysis of miRNA microarray data. The dots represent the differentially expressed miRNAs selected from 2,006 miRNA probes. PCA, principal component analysis; microRNA, miRNA.

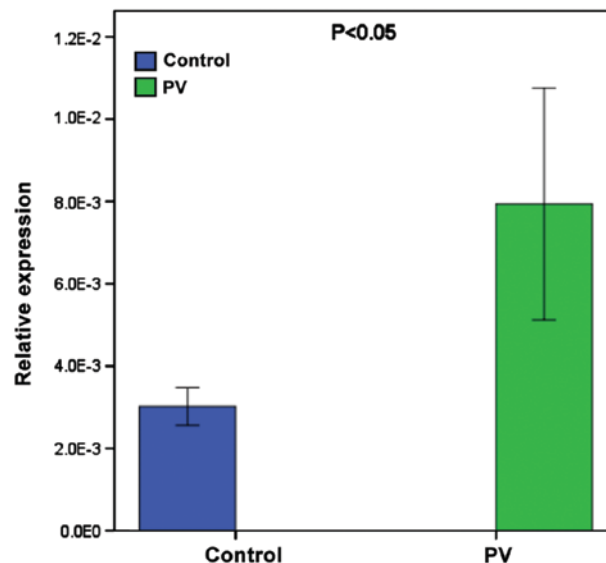


Figure 2. Validation of higher levels of miR-424-5p expression in patients with pemphigus. The relative levels of miR-424-5p normalized to U6 expression in PBMC from 9 patients with pemphigus and 9 healthy subjects were determined by qRT-PCR. Data are expressed as mean \pm SD of each group. PBMC, peripheral blood mononuclear cell; SD, standard deviation.

Table II. Biological process and functional annotation of hsa-miR-424-5p analyzed by GO.

GO_ID	Term	Count of genes	P-value	Benjamini
GO:003556	Intracellular signal transduction	242	7.9E-10	3.3E-6
GO:0051174	Regulation of phosphorus metabolic process	110	1.7E-8	3.5E-5
GO:0019220	Regulation of phosphate metabolic process	110	1.7E-8	3.5E-5
GO:0042325	Regulation of phosphorylation	106	2.7E-8	3.7E-5
GO:0043549	Regulation of kinase activity	85	9.4E-8	9.7E-5
GO:0045859	Regulation of protein kinase activity	82	1.8E-7	1.4E-4
GO:0051338	Regulation of transferase activity	85	6.1E-7	4.2E-4
GO:0006796	Phosphate metabolic process	183	7.4E-7	4.4E-4
GO:0006793	Phosphorus metabolic process	183	7.4E-7	4.4E-4
GO:0006468	Protein amino acid phosphorylation	133	1.4E-6	7.0E-4
GO:0007264	Small GTPase mediated signal transduction	72	1.4E-6	6.4E-4
GO:0035556	Protein kinase cascade	81	6.5E-6	2.7E-3
GO:0016055	Wnt receptor signaling pathway	36	4.8E-5	1.8E-2
GO:0006812	Cation transport	107	6.0E-5	2.1E-2
GO:0001932	Regulation of protein amino acid phosphorylation	43	7.0E-5	2.2E-2
GO:0016310	Phosphorylation	145	8.1E-5	2.4E-2
GO:0033674	Positive regulation of kinase activity	53	8.9E-5	2.4E-2
GO:0006811	Ion transport	139	1.2E-4	3.1E-2
GO:0000165	MAPK cascade	44	1.5E-4	3.5E-2
GO:0030001	Metal ion transport	91	1.5E-4	3.4E-2

P-value <0.05, Benjamini <0.05. GO, Gene Ontology; MAPK, mitogen-activated protein kinase.

Table III. KEGG pathway analysis of miR-424-5p.

KEGG pathway term	Count of genes	P-value	Benjamini
Insulin signaling pathway	43	5.4E-8	1.0E-5
Pathways in cancer	74	4.1E-6	3.8E-4
Neurotrophin signaling pathway	35	2.3E-5	8.5E-4
Wnt signaling pathway	39	6.2E-5	1.9E-3
mTOR signaling pathway	19	8.0E-5	2.1E-3
T cell receptor signaling pathway	29	3.4E-4	4.8E-3
GnRH signaling pathway	27	3.7E-4	4.9E-3
Fc epsilon RI signaling pathway	23	4.1E-4	5.1E-3
ErbB signaling pathway	24	8.3E-4	8.6E-3
MAPK signaling pathway	52	4.8E-3	3.8E-2

KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase.

humans. GO analysis indicated the functional categorization of predicted target genes. There were 20 biological processes ($P < 0.05$, Benjamini < 0.05) (12), including intracellular signaling cascades, phosphate metabolism and regulation of kinase activity (Table II). There were 242 potential target genes involved in the process of intracellular signal transduction. Functional annotation of miR-424-5p was analyzed by KEGG pathway analysis. The target genes were enriched in 10 signaling pathways ($P < 0.05$, Benjamini < 0.05), including the T-cell receptor signaling pathway and mitogen-activated protein kinase (MAPK) signaling pathway, suggesting that

miR-424-5p may influence these signaling pathways through target genes (Table III and Fig. 3).

Discussion

The present study examined the miRNA expression profiles in PBMCs from patients with pemphigus and healthy subjects by miRNA microarray analysis. We identified 124 differentially expressed miRNAs, 71 of which were upregulated and 53 of which were downregulated. Interestingly, we found higher levels of miR-424-5p expression in PBMCs from patients with

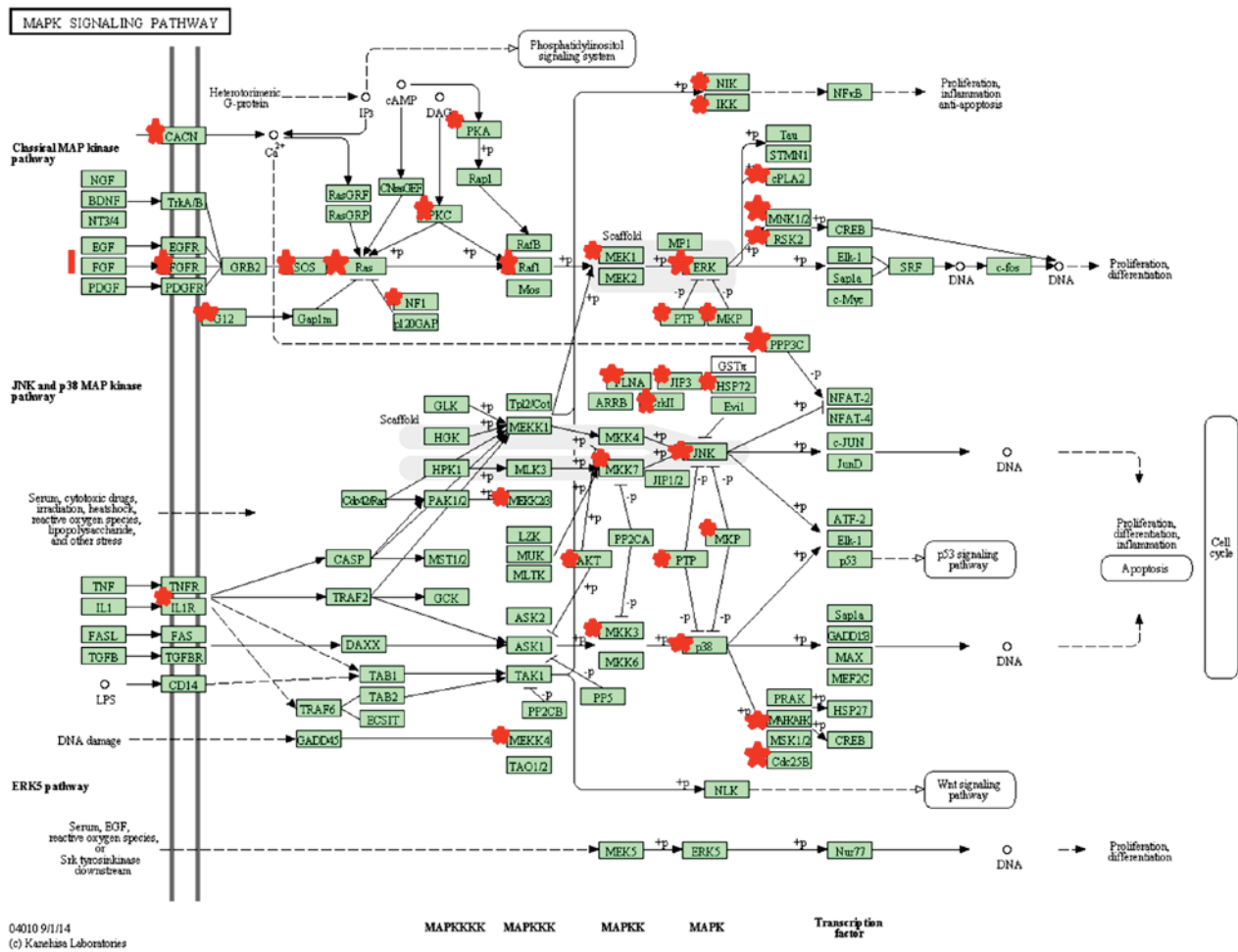


Figure 3. The MAPK signaling pathway. Genes with red stars are targeted by miR-424-5p. MAPK, mitogen-activated protein kinase.

pemphigus. We also described the biological function and regulation network of miR-424-5p, which may help explore the regulatory role of miR-424-5p in the pathogenesis of pemphigus.

Previous studies reported that miR-424-5p expression was dysregulated and correlated with age-related macular degeneration (13), endometriosis (14), cancer (15,16) and *Mycobacterium tuberculosis* infection (17). The difference is likely due to the different biological processes regulated by miR-424-5p in different tissues and organs. However, our data are in disagreement with a previous study that miR-424-5p expression was significantly decreased specifically in psoriasis skin. Downregulation of miR-424-5p expression leads to over-expression of the target genes MEK1 and cyclin E1, causing the hyper-proliferation of keratinocytes (18). Furthermore, miR-424 promotes monocyte differentiation, together with miR-155, miR-222 and miR-503 (19). The difference may stem from the different types of cells examined.

We analyzed the potential function of miR-424-5p by bioinformatics and found many potential target genes in functionally related groups. GO and KEGG analysis indicated that the biological function of the potential genes targeted by miR-424-5p were enriched in intracellular signaling cascades, phosphate metabolism and regulation of kinase activity. These potential target genes may regulate a wide range of pathways, such as the p38 and other MAPK signaling pathways. The MAPK signaling pathway is dysregulated

in the pathogenesis of pemphigus (20). miR-424-5p may regulate the pathogenesis of pemphigus by targeting the MAPK signaling pathway. A previous study showed that p38 MAPK is important for regulating autoimmune responses, cell survival, proliferation, differentiation and apoptosis (21). Activation of p38 MAPK was associated with collapse of the cytoskeleton, disassembly of desmosomes, and keratinocyte apoptosis (22). Pemphigus-specific autoantibodies can induce p38 MAPK activation in human keratinocytes and mouse keratinocytes, and inhibition of p38 MAPK activity can prevent pemphigus-specific, autoantibody-induced cytoskeletal reorganization, and heat shock protein (HSP) 27 phosphorylation in human keratinocytes (23). Furthermore, treatment with p38 inhibitors prevented skin blistering by inhibiting pemphigus IgG-activated signaling in epidermal cells in a mouse model of pemphigus induced by adoptive transfer of pemphigus-specific antibodies (24,25). Thus, the p38 MAPK signaling pathway is crucial for the pathogenesis of pemphigus. In addition, HSP27 participates in the pathogenesis of pemphigus and phosphorylated HSP27 is observed in skin biopsies from patients with pemphigus, mouse pemphigus models and cultured keratinocytes. HSP27 phosphorylation has been considered to be involved in regulation of the cytoskeletal assembly of actin filaments and keratin intermediate filaments. Dysregulated cytoskeletal arrangements are associated with the loss of cell-cell adhesion, and it is possible that

miR-424-5p regulates p38 MAPK activation and HSP27 phosphorylation, and blister formation during the pathogenesis of pemphigus (23,25,26).

We predicted that there were 52 target genes of miR-424-5p enriched in the MAPK signaling pathway. This indicates that miR-424-5p may regulate p38 MAPK activation and HSP27 phosphorylation in human PBMCs. miR-424-5p may regulate HSP27 phosphorylation by targeting the upstream MAPKAPK3 and CDC25B. MAPKAPK3 is a member of the Ser/Thr protein kinase family. This kinase can be activated by growth factors and stress stimuli, and is involved in cell responses and gene regulation (27). CDC25B is a member of the CDC25 phosphatase family and is regulated by the p38 MAPK and/or MAPKAP kinase-2 pathways (28,29). The dysregulated expression of MAPKAPK3 is associated with the development of various types of cancer, but its specific role in tumor formation remains to be determined (30-32). Further investigations are required focusing on the exact roles of MAPKAPK3 and CDC25B in the pathogenesis of pemphigus.

In conclusion, we examined the miRNA expression profile of PBMCs from patients with pemphigus by miRNA microarray analysis. To the best of our knowledge, we are the first to identify differentially expressed miRNAs in PBMCs from patients with pemphigus and demonstrated higher levels of miR-424-5p in PBMCs from these patients. A bioinformatics approach predicted the potential target genes, biological functions, and pathways of miR-424-5p, indicating that miR-424-5p may contribute to the pathogenesis of pemphigus by regulating the p38 MAPK signaling pathway. The influence of miR-424-5p on HSP27 phosphorylation by targeting MAPKAPK3 and CDC25B may also regulate the pathogenesis of pemphigus.

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