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Combining AlphaFold—based AI docking with omics technologies preliminarily unveils the mechanism of Guominkang in treating allergic rhinitis

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Allergic rhinitis (AR) is a common immune-mediated chronic inflammatory disease with a complex pathogenesis involving multiple responses of the immune system and epigenetic changes. In recent years, DNA methylation, a key epigenetic mechanism, has been shown to play an important role in the onset and development of AR. GuoMinKang (GMK) have been used in the treatment of AR through their multi-component properties. However, its specific epigenetic regulatory mechanisms have not been fully investigated. The aim of this study was to explore the epigenetic regulatory mechanisms of the traditional Chinese medicine compound GMK in the treatment of AR. By analysing DNA methylation and transcriptome data from AR patients, we identified differentially methylated regions (DMRs) and differentially expressed genes (DEGs) associated with AR. Through network pharmacology analysis, we screened the active components of GMK and their potential target genes, particularly those related to DNA methyltransferases (DNMTs). An AR mouse model was established to observe the behavioural and pathological changes of the nasal mucosa of mice after drug administration; the expression of IgE cytokines was detected by ELISA, and the expression of nasal mucosa genes was verified by qPCR. Total IgE (tIgE) levels were significantly reduced in AR patients after GMK treatment, suggesting a possible role in immunomodulation. Our analysis revealed that GMK was able to restore aberrant methylation patterns in AR patients by modulating specific DNA methylation regions. Through differential methylation analysis, we identified 10 genes whose methylation levels were significantly restored to normal after GMK treatment and which already showed significant differential expression in AR patients, particularly in immune regulation and epithelial cell function. These genes include LERP, NFIA, etc., suggesting that they may play a key role in the onset and development of AR. Further through target prediction and network pharmacological analyses, we confirmed that the active ingredients of GMK (e.g., quercetin, coumarin, and geranylgeranyl) may exert their epigenetic regulatory functions by targeting the protein activity of DNMTs. In vivo experiments showed that GMK reduced the number of nose scratching and sneezing in mice, glandular hyperplasia in the nasal mucosa was alleviated, with a reduction in the number and volume of glands, and serum tIgE levels were reduced. The increase in LERP expression in the AR model was reduced after treatment with GMK, and the change in NFIA expression was not significant. It suggests that GMK may regulate LERP activity through DNMTs to alleviate allergic symptoms. This study reveals the potential therapeutic mechanism of the traditional Chinese medicine compound GMK in regulating AR through epigenetic mechanisms. These findings provide a theoretical basis and molecular foundation for the clinical application of GMK in AR and open up new research directions.

Keywords Allergic rhinitis, DNA methylation, GMK, DNMTs, Immunity

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AR is an upper respiratory tract disease, a non-infectious chronic inflammatory disease of the nasal mucosa mediated by immunoglobulin E (IgE). AR affects more than 400 million people worldwide, making it one of the most prevalent chronic diseases[1], and it imposes a significant health burden on individuals, affects quality of life, and is associated with severe comorbidities, including asthma, sinusitis, and conjunctivitis. AR is the result of a type I hypersensitivity reaction occurring within the nasal mucosa in response to airborne allergens such as grass and tree pollens, house dust mites and animal dander. The main symptoms are nasal congestion, watery rhinorrhea, pruritus and paroxysmal sneezing[2]. AR is a complex disease with an etiology that involves genotype, epigenetics (including DNA methylation), environmental factors, climate change and lifestyle, and the mechanisms involved include oxidative stress on the airways, triggering inflammation, structural remodelling and increased risk of sensitisation[3,4]. In particular, an increase in environmental air pollutants has been found to be associated with an increased incidence of allergic respiratory diseases, and evidence suggests that exposure to ambient particulate matter (PM2.5) may increase DNA methylation in vivo[4,5].Therapeutic options for AR include allergen avoidance, treatment with steroids or antihistamines, immunotherapy, and surgical intervention[2,6,7]. The rationale for AR and the therapeutic mechanisms have been extensively studied, but many aspects remain unclear and require further research.

In the clinic, the short-term effect of western medical treatment of AR is remarkable, but the efficacy is difficult to be sustained and easy to relapse after stopping the drug[8]. In addition, hormones and other drugs are highly dependent, and antihistamines have side effects such as drowsiness, making them unsuitable for long-term use[9]. With their multi-components and mild modifying effects, GMK have demonstrated unique advantages in the treatment of chronic diseases and have been widely used in the treatment of AR with significant efficacy and good safety. In the field of Chinese medicine research, the pathogenesis of allergic rhinitis can be categorised as dysfunction of the internal organs, including deficiency of lung qi, spleen qi, or deficiency of kidney yang leading to wind-cold obstruction; therefore, TCM treats both the symptoms and the root cause[10]. Currently, studies on the treatment of AR with herbal medicines have focused on the modulatory effects on oxidative stress, inflammation and immunity[11]. For example, Nasal Abyss Tongjiao Granules inhibited the expression of *E-selectin* and *VCAM-1* and *ICAM-1* by modulating *PI3K/AKT* and *STAT3/MAPK* signalling pathways, thereby reducing inflammation in AR mice[12]. Xiaoqinglong Tang regulates *ILC2* via *IL-33/ST2* and *JAK/STAT* pathways to ameliorate type 2 inflammation in ovalbumin (OVA)-induced AR[13].

DNA methylation (DNAm) has been extensively studied in living organisms as an important epigenetic modality of regulation and has been linked to biological topics such as gene regulation and genome organisation, reproduction and development, as well as disease and aging, making it the most well-studied epigenetic mechanism[14,15]. DNA methylation is the process of adding methyl groups to cytosine nucleotides in the cytosine-phosphate-guanine (CpG) dinucleotide sequence. These CpG sites are usually clustered in "CpG islands" within gene regulatory elements such as promoters or enhancers to affect transcription. In recent years, advances in the study of allergic diseases have shown that DNA methylation plays a crucial role in their pathogenesis[16–19]. T cells, B cells, macrophages and mast cells are involved in the evolution and progression of allergic diseases, and DNA methylation affects the behaviour of these immune cells. For example, DNAm patterns are altered within CD4 + T cells in AR[20]. In addition, TET proteins and 5-hydroxymethylcytosine mediate immune response mechanisms in AR[16]. It has also been shown that *SLFN12* DNA methylation is significantly associated with AR symptoms; changes in *MUC4* DNA methylation in nasal brushes correlate with a reduction in peak nasal inspiratory flow[4]. Reductions in DNA methylation attenuate the allergic symptoms of AR and normalise an overreactive immune system[19].

Traditional Chinese medicine (TCM) significantly affects a wide range of diseases through epigenetic mechanisms, such as DNA methylation. For example, Liuwei Di Huang Wan prevented postmenopausal atherosclerosis and endothelial cell apoptosis[21]. The Phenomenon of the Free Pill rescued aberrant gene expression and DNA methylation levels associated with depression by up-regulating DNMT1[22]. Bazi tonic formula targets the *SIRT3-FOXO1* pathway and *ERCC2* through methylation regulation and exerts anti-aging effects[23]. Chinese medicine plays an important role in anti-tumour by regulating the process of DNA methylation modification. The study of Chinese medicine in epigenetic regulation is an emerging and important field. The theory of Chinese medicine suggests that diseases originate from the imbalance of yin and yang balance in the human body, and Chinese medicine achieves therapeutic effects by regulating the state of yin and yang imbalance. The reversibility of epigenetic mechanisms provides a scientific basis for this theory. Nevertheless, the multi-targeted mechanism of action of TCM has not yet been fully clarified, and relatively few studies have been conducted on its regulatory role at the epigenetic level, especially in AR, which has not yet been reported. In view of the key role of DNA methylation in the pathogenesis of AR, further studies on the specific effects of the TCM compound GMK on DNA methylation and its mechanism of action in the treatment of AR are necessary.

In this study, we focused on the epigenetic role of the traditional Chinese medicine compound GMK in the treatment of AR. First, by analysing DNA methylation data from AR patients before and after treatment with GMK, we identified genes that significantly restored the normal methylation status and explored the role of these genes in immune regulation and epithelial cell function. In particular, we found that GMK was able to reverse the aberrant methylation pattern in AR patients by modulating specific DNA methylation regions, improving immune cell ratios and significantly reducing IgE levels. Further transcriptome analyses revealed that the therapeutic effect of GMK is not only in methylation repair, but also by regulating the expression of immune-related genes, which may restore immune homeostasis and alleviate the symptoms of AR. Through network pharmacological analyses, we revealed the interaction network of the active ingredients in GMK with epigenetic targets such as DNMTs, providing a new molecular basis for the potential epigenetic regulatory mechanisms

of GMK in AR therapy. To visually illustrate the potential binding targets of GMK's active components, we supplemented our analysis with AI-driven structural predictions generated by AlphaFold, a tool renowned for its high-precision protein structure modelling[24]. However, subsequent molecular docking simulations employed experimentally validated structures from the Protein Data Bank (PDB) to ensure reliability. Finally, preliminary validation was carried out by animal experiments. These findings provide a theoretical basis for the clinical application of GMK and a new research perspective for its intervention in the treatment of allergic rhinitis through epigenetic mechanisms.

Materials and methods

Sample collection

Inclusion criteria included individuals between the ages of 18 and 60 years who were diagnosed with allergic rhinitis and exhibited at least two symptoms of AR. Exclusion criteria included individuals with comorbidities such as asthma, allergic diseases, chronic sinusitis, and other immune or major organ diseases. Pregnant or breastfeeding women were excluded, as well as individuals with poor compliance or participation in other clinical trials. Exclusion criteria included enrolment errors, poor compliance, adverse reactions or complications. The Total Nasal Symptom Score (TNSS) served as the primary outcome measure. Participants in the AR group received GMK treatment for a duration of 8 weeks. The baseline characteristics of the participants are summarized in Table S1.

Human ethics approval

The trial adhered to the Declaration of Helsinki (Edinburgh, 2000). It was approved by the Medical Ethics Committee of Beijing University of Chinese Medicine (ID: 2023BZYLL0109) and the Ethics Management Committee of Nantong Hospital, Shanghai University (ID: 2022025). The study was registered at ClinicalTrials.gov. on February 9, 2023 (ID Number: ChiCTR2300068155). Participants provided written informed consent before enrollment.

Peripheral blood mono-nuclear cell (PBMC) separation

Take 2 mL of EDTA anticoagulated blood from the subject, dilute it with equal volume of PBS, separate it with lymphocyte separator, add the diluted anticoagulated blood to the top of the separator according to the instructions, the volume ratio is 1:1, centrifuge at 800 g for 20 min, extract the white membrane layer, which is PBMC.

Identification of differentially methylated regions (DMRs)

Data processing was conducted using the ChAMP package (version 2.26.0) and the IlluminaHumanMethylationEPICv2manifest package (version 0.1.0). The data was imported using the read.metharray() function from ChAMP, with probe annotation information provided by IlluminaHumanMethylationEPICv2manifest. Differential DNA methylation analysis was performed using the ChAMP package in R. Methylation levels at CpG sites were quantified, and differentially methylated probes (DMPs) between groups were identified using the statistical models provided by ChAMP, which account for multiple testing and confounding factors. Significant DMPs were then aggregated into DMRs using the DMRcate package. DMRs were classified as hypermethylated or hypomethylated in AR compared to healthy controls (HC).

To identify DMRs indicative of epigenetic restoration by GMK, we first identified hypermethylated and hypomethylated regions in AR patients compared to HC. Subsequently, we compared the GMK group with the AR group to identify regions where methylation levels were reversed. Specifically, hypermethylated DMRs in AR vs. HC were overlapped with hypomethylated DMRs in Treatment vs. AR, and hypomethylated DMRs in AR vs. HC were overlapped with hypermethylated DMRs in Treatment vs. AR. These overlapping DMRs were considered to have their methylation status restored toward normal levels following TCM treatment, suggesting potential epigenetic restoration effects of GMK.

Retrieval and process of public data

Public RNA-seq data related to AR were obtained from the Sequence Read Archive (SRA) under the accession number SRP548490. A total of 10 RNA-seq datasets, including 5 healthy controls and 5 untreated AR patients, were downloaded. The SRA Run files were converted to FASTQ format using the NCBI SRA Toolkit (fastq-dump). Raw reads were trimmed of low-quality bases and adapter sequences using the FASTX-Toolkit (v0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/). The quality of the cleaned reads was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc).

Identification of differentially expressed genes (DEGs)

Differential gene expression analysis was performed using DESeq2[25], a widely adopted software package for analyzing count-based RNA-seq data. Raw count data were input into DESeq2 to identify DEGs between AR patients and healthy controls. DEGs were determined based on a threshold of $|\text{Fold Change}| > 1.5$ and a P-value ≤ 0.05. Genes meeting these criteria were classified as significantly upregulated or downregulated in AR compared to healthy controls.

Cell type quantification

The CIBERSORT algorithm[26] (v1.03) was used with the default parameter for estimating immune cell fractions using FPKM values of each expressed gene. A total of 22 human immune cell phenotypes were analyzed in the study, including seven T cell types [CD8 T cells, naive CD4 T cells, memory CD4 resting T cells, memory CD4 activated T cells, T follicular helper cells, and regulatory T cells (Tregs)]; naive and memory B cells; plasma cells;

resting and activated NK cells; monocytes; macrophages M0, M1, and M2; resting and activated dendritic cells; resting and activated mast cells; eosinophils; and neutrophils.

Functional enrichment analysis

To identify functional categories of genes, we employed the clusterProfiler package (v4.6.2)[27], which enabled us to determine Gene Ontology (GO) terms and KEGG pathways[28,29].

Overlap and correlation analysis of differentially methylated and expressed genes

To identify genes potentially regulated by methylation changes due to GMK treatment, we performed an overlap analysis between DMRs restored to normal levels following GMK treatment and DEGs associated with AR. To explore the relationship between these genes' expression levels and immune cell proportions, Spearman correlation analysis was conducted. The expression levels of the overlapping genes were correlated with the proportions of various immune cell types estimated by CIBERSORT. These correlations were visualized using the ggplot2 and corrplot packages in R, highlighting significant positive or negative associations between gene expression and immune cell proportions.

Traditional Chinese medicine Pharmacology network analysis

The chemical constituents of GMK identified through HERB database were filtered based on Lipinski's rule of five and bioavailability scores > 0.2 using SwissADME (http://www.swissadme.ch). Metabolite information, including compound IDs (CIDs) and SMILES notations, was retrieved from PubChem using RESTful API calls. ChEMBL IDs were obtained via the UniChem API, and the targets for each metabolite were compiled from PubChem and ChEMBL databases, filtered to include only Homo sapiens targets. After applying Lipinski's "rule of five," compounds that passed the bioavailability criteria were kept, and target prediction was performed, resulting in 141 compounds with identified targets. The targets of GMK compounds related to DNA methylation were selected. Cytoscape software was used to construct a "herb-compound-target gene" interaction network, illustrating the pharmacological mechanisms of GMK, particularly its potential epigenetic regulation via DNMTs.

Molecular docking analysis

Key bioactive compounds identified from GMK were subjected to molecular docking analysis to predict interactions with constipation-related protein targets. Three-dimensional structures of the compounds were obtained from the pubChem database and converted to pdbqt format using OpenBabel (v3.1.1). Protein structures of DNMT enzymes were downloaded from the Protein Data Bank (PDB) and processed using MGLTools (v1.5.7) by removing water molecules, adding hydrogen atoms, and assigning charges.

Docking simulations were conducted using AutoDock Vina (v1.2.5) to calculate binding affinities, with compounds ranked by the lowest binding energy scores. PyMOL (v3.0.3) was employed to visualize docking poses and generate interaction diagrams, highlighting critical binding sites and interactions between compounds and target proteins.

Animal models of AR and GMK Preparation

BALB/c female mice (6–8 weeks old) were purchased from Beijing Spefo Laboratory Animal Technology Co. and were housed in a specific pathogen free (SPF) environment. All animal experiments followed the standards of the Animal Care and Use Committee of Beijing University of Chinese Medicine, AAALAC and IACUC. Mice were randomly divided into three groups of 10 mice each: blank control group (control), model group (model), and GMK-treated group (GMK). AR mice were sensitised by intraperitoneal injections of 100 μg of OVA (Sigma-Aldrich, St. Louis, USA) and 4 mg of hydroxide (Thermo Fisher Scientific, Waltham, MA, USA) on days 0, 7, and 14, and stimulated once daily with 20 μL of 800 μg of OVA administered intranasally from day 21 to day 27, during which time they were treated with GMK. GMK consists of Wu-Mei, Chan -Tui, Ling-Zhi and Fang-feng, which were purchased and soaked in deionised water for 30 min, then decocted at 100 °C. The ingredients were then added to a volume of deionised water and decocted once more. The decoction was repeated with the same volume of deionised water under the same conditions. From day 14 to day 27, 19.5 g/kg of GMK was administered by gavage once daily[30]. The control group was treated accordingly with equal amounts of saline. For the control group, 24 h after the last stimulation, mice were anaesthetised using 2% isoflurane. Once the mice were fully anaesthetised, blood was collected via orbital puncture. The mice were then euthanised by cervical dislocation, and nasal mucosa samples were collected for further analysis.

Animal ethics approvals

This study adheres to internationally accepted standards for animal research, following the 3Rs principle. The ARRIVE guidelines were employed for reporting experiments involving live animals, promoting ethical research practices. Meanwhile, all animal experiments have been approved by the Institutional Animal Ethics Committee of the Beijing University of Chinese Medicine in China (Ethics approval No. BUCM-1-2020102701-0010).

Behavioural observations of mice

At the end of the nose drops, experimental recordings were made to record the symptoms of nose scratching and sneezing, and after the mice had rested for at least 5 min, the number of times a single mouse developed both symptoms in a 15-minute period was recorded in a single-cage observation, and the data obtained were expressed as the average number of nose scratches/sneezes per minute. Mice in the blank control group were given saline instead of OVA.

Pathological tissue staining

Nasal mucosa/turbinates were fixed in 4% paraformaldehyde solution for 24 h, then subjected to HE staining and PAS staining (Wuhan Safe Biotechnology Co., Ltd., China). HE staining: paraffin sections were deparaffinised, stained with hematoxylin-eosin, dehydrated and then sealed, and the nuclei of the cells appeared to be blue to bluish-purple and the cytoplasm appeared to be reddish under the microscope. PAS staining: the sections were deparaffinised, acidified with periodate, rinsed with water and then stained with PAS.

Enzyme-linked immunosorbent assay (ELISA)

Collect mouse serum and perform the assay according to the manufacturer's instructions (Cayman Chemical, product number 500840). Add 100 µL of dilution standard to the reaction wells and 100 µL of sample to be tested to the reaction wells. After 2 h of incubation, add 100 µL of biotin-labelled antibody. Cover the membrane plate, shake gently and incubate at 37 °C for 1 h. Shake off the liquid in the wells, add 100 µL of substrate to each well, shake gently and incubate at 37 °C for 30 min, avoiding light. Remove the plate and quickly add 100 µL of termination solution, the results should be measured immediately after the addition of termination solution. Determine the OD value of each well at 450 nm.

RT-qPCR

Total RNA was isolated using a total RNA extraction reagent (Tiangen, China) and reverse transcription was performed using First-strand cDNA Synthesis Mix (LABLEAD, China) according to the manufacturer's instructions. The cDNA was detected using SYBR Green qPCR Master Mix (LABLEAD, China) and a real-time fluorescence PCR instrument (Bio-rad, USA), and the experiment was repeated three times independently, and the fold change was calculated by the 2-ΔΔCT method.

Statistical analysis

Principal component analysis (PCA) analysis was performed by R package factoextra (https://cloud.r-project.org/package=factoextra) to show the clustering of samples with the first two components. After normalizing the reads by TPM (Tags Per Million) of each gene in samples, in house-script (sogen) was used for visualization of next-generation sequence data and genomic annotations. The pheatmap package (https://cran.r-project.org/web/packages/pheatmap/index.html) in R was used to perform the clustering based on Euclidean distance.

The data were statistically analysed using SPSS 26.0 statistical software. Experimental results were obtained from three independent replicates. Normality and chi-square tests were performed on all data. The results of each group were expressed as mean earth SEM. Depending on the type of data, differences between groups were analysed using unpaired t-tests or chi-square tests, while multiple comparisons were made using one-way or multi-factor ANOVA. Statistically significant differences were indicated by a P value < 0.05. GraphPad Prism 10.0 software was used to draw statistical plots.

Results

Analysing the workflow

In order to explore the epigenetic mechanism of AR treated with GMK, we first collected samples from the healthy control group, the AR patient group, and the GMK-treated group based on the inclusion criteria (Fig. 1A), and identified methylation differences between the disease group and the treatment group by DMR analysis, with a special focus on genes that regained their normal methylation after the treatment with GMK. Subsequently, we downloaded and analysed transcriptome data from peripheral blood mononuclear cells (PBMCs) of AR patients via the GEO database (selecting published data from Southwest Medical University, where subjects shared identical ethnic backgrounds and the dataset comprised identical samples), screened for DEGs and further explored the changes in the proportion of immune cells. In order to explore how GMK therapy affects gene expression and immune response through epigenetic changes, we screened key targets by correlation analysis of differentially methylated genes and differentially expressed genes regulated by GMK therapy, and anticipated the regulatory effects of these genes on immunity through correlation analysis with immune cells. Finally, we analysed the GMK by network pharmacology, predicted the targets of its active ingredients, and constructed GMK compound-active molecule-target gene regulatory network, which revealed that GMK may restore the aberrant methylation pattern of AR by regulating epigenetic mechanisms such as DNMTs (Fig. 1B). Further, we measured the tIgE levels of the patients before and after treatment (Fig. 1C), which showed a significant decrease in tIgE levels after treatment, suggesting that GMK treatment may alleviate the symptoms of allergic rhinitis by modulating the immune response, especially by decreasing the synthesis of IgE, which further supports the potential role of TCM in immunomodulation.

Identification of differentially methylated genes restored to normal levels in patients with allergic rhinitis after treatment with GMK

To elucidate the epigenetic regulatory mechanism of GMK in the treatment of AR, we conducted a comprehensive analysis of DNA methylation patterns in a healthy control group, an untreated AR patient group, and an AR patient group treated with GMK. The results of PCA analysis based on methylation levels showed that the second principal component clearly distinguished the healthy control group from the untreated AR patient group and the GMK treatment group. Further, the first principal component effectively distinguished the untreated AR patient group from GMK treatment group, indicating that both AR pathology and GMK intervention significantly altered DNA methylation patterns (Fig. 2A). The number of DMRs increased significantly after GMK treatment, especially Hypo-DMRs (regions with decreased methylation levels), suggesting that GMK has a strong regulatory effect on DNA methylation patterns (Fig. 2B). To understand the biological functions of the genes where these DMRs are located, we performed GO enrichment analysis. The results showed that the gene

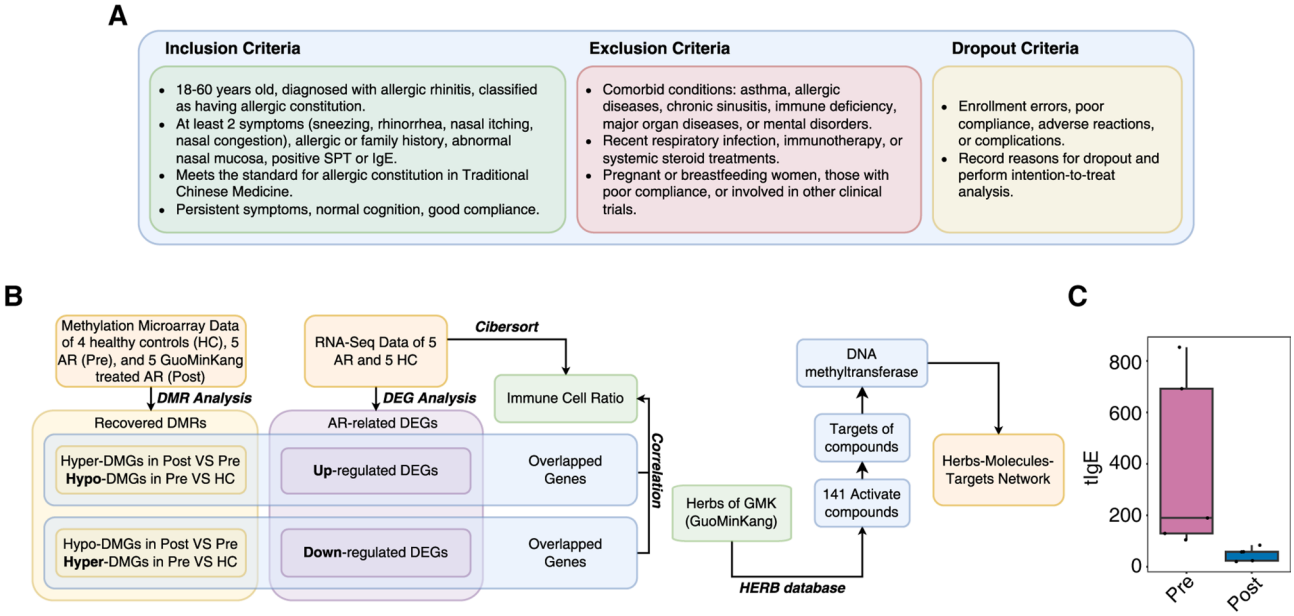

Fig. 1. Study design and analysis workflow. (**A**) Patient Inclusion, Exclusion, and Dropout Criteria. (**B**) Flowchart illustrates the workflow of bioinformatic analysis in this study. (**C**) Bar plot showing the change in tIgE levels before (Pre) and after (Post) treatment with GMK.

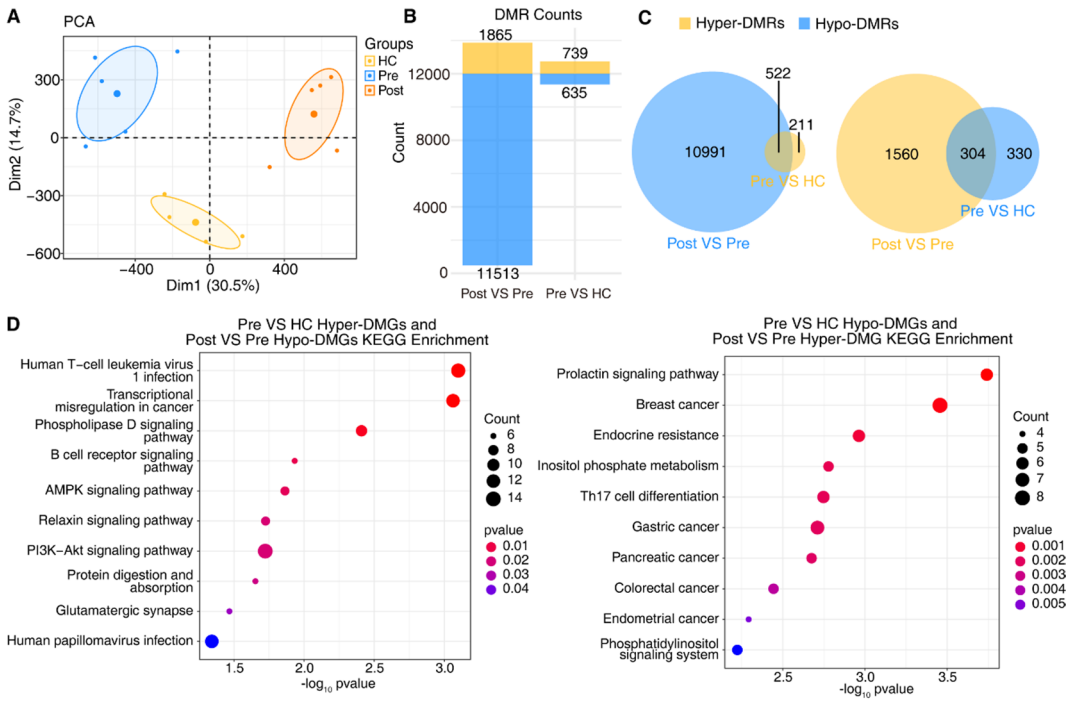

Fig. 2. Analysis of Differentially Methylated Regions and Genes in AR. (**A**) Principal component analysis (PCA) of DMPs beta values. (**B**) The differences in the number of DMRs across the comparison groups are illustrated in a bar plot. (**C**) Venn diagrams illustrating the overlap of hyper (hypo) methylated regions and hypo (hyper) methylated regions between different groups. (**D**) KEGG enrichment analysis of genes whose methylation levels were restored after GMK treatment.

function of DMRs with increased methylation levels in the AR disease group compared to the healthy group was associated with branching morphogenesis, epithelial tube morphogenesis, mature B cell differentiation, immune system development, and B cell receptor signalling pathway, while the gene function of DMRs with decreased methylation levels was associated with phosphatidylinositol 3 phosphate biosynthesis, epithelial cell proliferation,

myoblast differentiation, siRNA passage through hetero chromatin formation mediating retrotransposon silencing, cell junctions, etc. (Figure S1A). Compared to the AR disease group, the gene function of DMRs with increased methylation levels in the GMK-treated group was associated with lymphocyte differentiation, T-cell differentiation, neurodevelopment, monocyte differentiation, and synaptic organisation, and the gene function of DMRs with decreased methylation levels was associated with small GTPase-mediated signalling, projection neuron development, actin filament organisation, cell matrix adhesion, and glycerol ester metabolism related (Figure S1B).

To help investigate the mechanisms of GMK regulation of DNA methylation in AR, we focused on genes whose methylation levels were altered after treatment with GMK compared to the AR disease group. Therefore, by taking the intersection of Hyper-DMRs (regions with increased methylation levels) with Hypo-DMRs (regions with decreased methylation levels) after treatment and Hypo-DMRs in disease with Hyper-DMRs after treatment in the comparison of disease and healthy groups, respectively, we can get that there are 522 methylated regions whose methylation levels were decreased by GMK treatment, and there are 304 methylated regions whose methylation levels are increased by GMK treatment, and the methylation status of these regions may be normalised by GMK treatment (Fig. 2C). KEGG enrichment analysis showed that the biological functions of genes with reduced methylation levels after GMK treatment were associated with epithelial tube morphogenesis, monosaccharide metabolism, immune system development, mesenchymal development, and lymphocyte differentiation; whereas the functions of genes with increased methylation levels after GMK treatment were mainly enriched in the areas of epithelial cell proliferation and regulation, and siRNAs mediating retrotransposon silencing through heterochromatin formation, and retrotransposon silencing mediated by siRNA through heterochromatin formation (Fig. 2D). The above results suggest that GMK restored the aberrant methylation patterns in AR patients by modulating specific DNA methylation regions. These restored normal DMRs-related genes are involved in key immune regulation, epithelial cell function and metabolic pathways, suggesting that GMK may exert its therapeutic effects on AR through these pathways, improving the immune homeostasis and epithelial barrier function of the patients, and thus alleviating AR symptoms.

Analysis of differentially expressed genes and proportion of immune cells in allergic rhinitis transcriptome data

To gain insight into the mechanism of action of GMK in the treatment of AR, we downloaded and analysed PBMC transcriptome sequencing data (SRP548490) from patients with allergic rhinitis, including five from healthy controls and five from AR patients. Based on principal component analysis (PCA), Fig. 3A showed significant differences in gene expression patterns between the healthy control group and the AR patient group, indicating that AR has a significant effect on gene expression. By volcano plot analysis (Fig. 3B), we identified significantly up-regulated DEGs including *RAB15*, *U2AF1L5*, *HES4*, *ABHD4*, *RP11-274A11.3*, *MFAP1*, *LINC00671*, *NEIL3*, *FUT10*, and *H4C5* in AR patients, and significantly down-regulated DEGs such as *ZRANB2*, *IL6ST*, *ACVR1C*, *HOOK1*, *ABCD2*, *THEMIS*, *ELP4*, *ZDBF2*, *CHML* and *ZNF260*. Subsequently, GO enrichment analysis of these DEGs (Fig. 3C) showed that up-regulated DEGs were mainly involved in the regulation of viral entry into host cells, regulation of biological processes of symbiotic interactions, nucleosome assembly and regulation of innate immune responses; while down-regulated DEGs were mainly associated with taste perception, cell polarity establishment and *Wnt* signalling pathway. In addition, the results of gene set enrichment analysis (GSEA, Fig. 3D) showed that the expression levels of gene sets associated with allograft rejection, inflammatory response, and *IFN-α* response were significantly increased in the AR group, which further demonstrated that the occurrence of AR was closely related to the abnormal response of the immune system. Finally, by immune cell ratio analysis (Fig. 3E), a significant decrease in the proportion of CD8 T cells and a significant increase in the proportion of neutrophils were found in patients with AR, suggesting that the imbalance in the proportion of immune cells may lead to over-activation of allergic reactions and exacerbation of inflammatory responses, which may trigger symptoms such as itchy, stuffy and runny nose. These results indicate that the gene expression profiles and immune cell ratios of AR patients are significantly different from those of healthy controls.

Correlation analysis of differentially methylated and differentially expressed genes in AR

To further explore the mechanism by which GMK regulates gene expression through methylation, we performed an overlap analysis of DMGs that returned to normal after GMK treatment with DEGs in the AR disease group to identify potential epigenetic regulatory targets. By Venn diagram analysis (Fig. 4A), we screened 10 overlapping genes, including *ESF1*, *ZRANB2*, *DCLK2*, *PEG10*, *DUSP19*, *LEPR*, *DYNC2H1*, *DAB2IP*, *NFIA* and *CTD-2260A17.2*. The expression of these genes in the AR group versus the healthy group is demonstrated by a box-and-line plot (Fig. 4B), in which *DAB2IP* and *CTD-2260A17.2* were up-regulated in the AR group, while the remaining eight genes were down-regulated in the AR group. Next, we performed a Spearman's correlation analysis of gene expression with the proportion of immune cells for these overlapping genes (Fig. 4C). The results showed that, except for *CTD-2260A17.2*, the expression levels of the remaining nine genes were significantly positively correlated with the proportion of CD8 T cells and significantly negatively correlated with the proportion of neutrophils. This finding is consistent with the significant decrease in the proportion of CD8 T cells and the significant increase in the proportion of neutrophils in AR patients in the previous section, further supporting the potential role of these overlapping genes in the treatment of AR with herbal medicine.

Identification of therapeutic targets and mechanisms of GMK for the treatment of allergic rhinitis by network pharmacological analysis

From the above studies, we found that GMK has a significant regulatory effect on DNA methylation, and we speculate that the active molecules in GMK may target DNA methylation enzymes, thereby affecting changes in DNA methylation patterns. Among these compounds, 512 compounds with PubChem IDs were included.

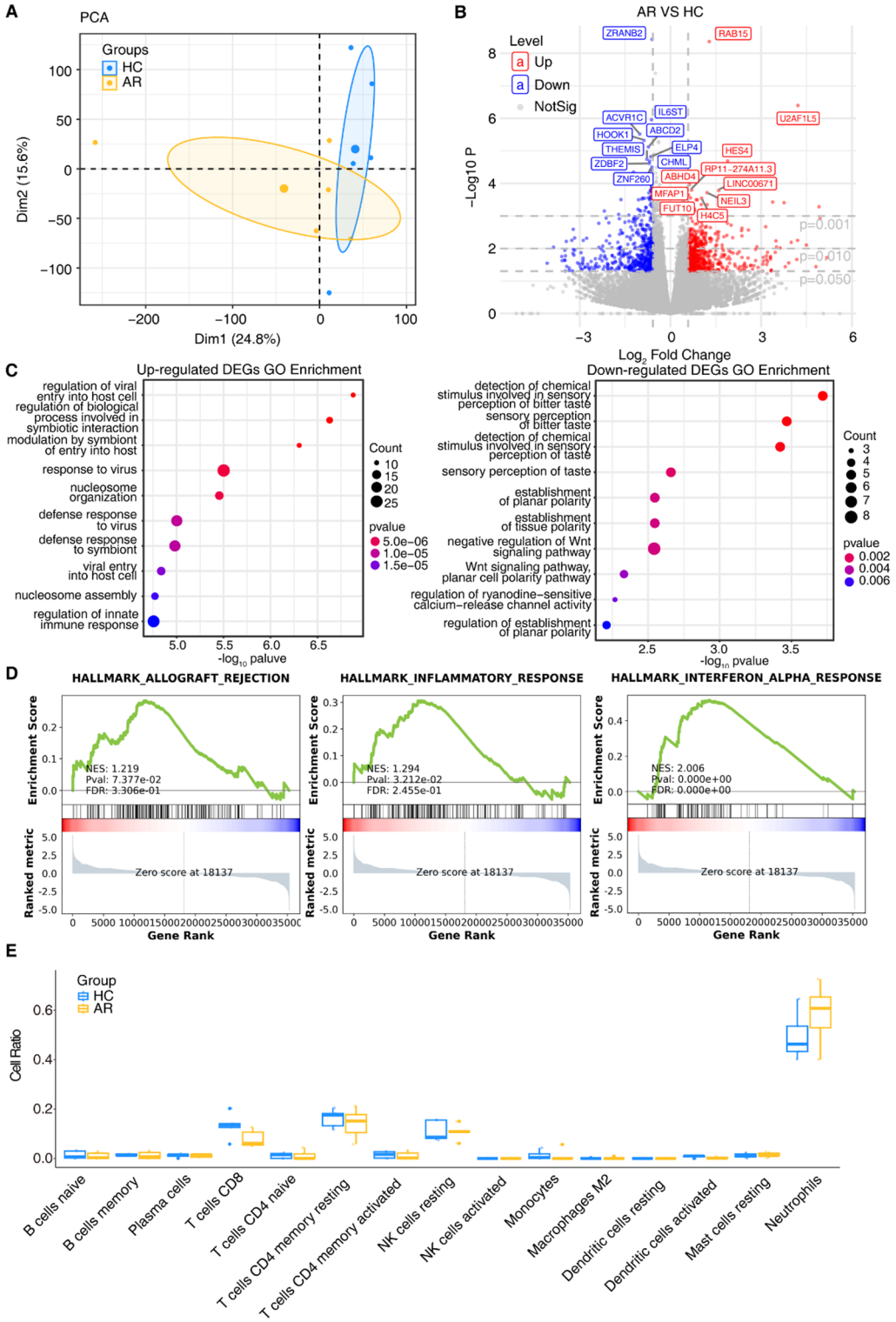

Fig. 3. Analysis of differentially expressed genes and immune cell ratio in the transcriptome data of AR. (**A**) PCA analysis of two sets of samples from AR PBMC transcriptome sequencing data (SRP548490). (**B**) Volcano plots demonstrating differentially expressed genes in the two sets of samples. (**C**) GO enrichment analysis of up-regulated and down-regulated differentially expressed genes. (**D**) GSEA results demonstrating significantly enriched biological pathways such as allograft rejection, inflammatory response and *IFN-α* response in the AR group. (**E**) Changes in the proportion of immune cells in samples from both groups.

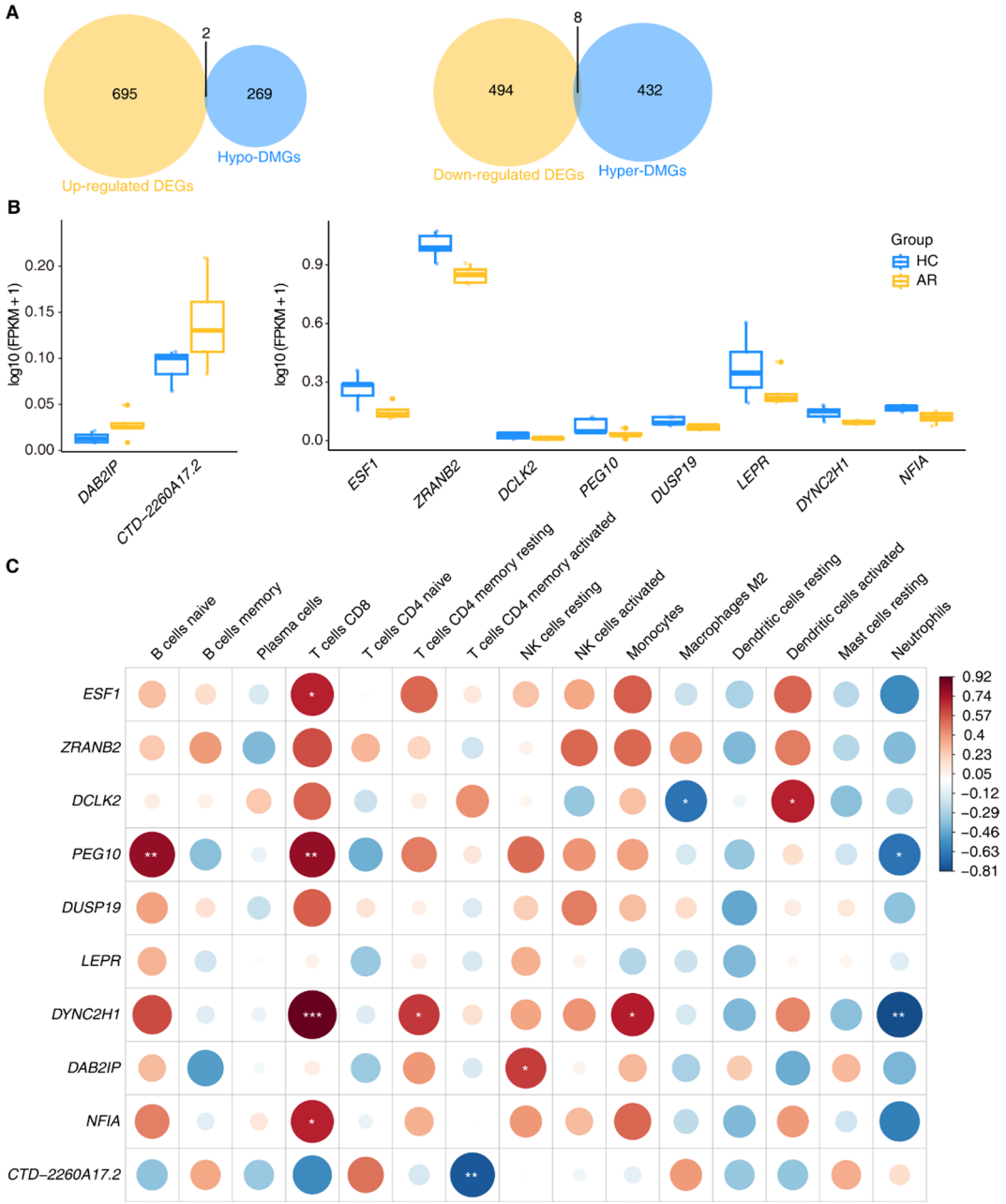

Fig. 4. Correlation analysis between DEGs affected by methylation and the proportions of immune cell types. (**A**) Venn diagram demonstrating the results of overlap analysis with differentially methylated genes that returned to normal after GMK treatment. (**B**) Box line plots showing the expression of intersecting genes in samples from the AR group and the healthy group. (**C**) Plot of Spearman correlation analysis based on gene expression versus immune cell ratio. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

We screened the compounds according to Lipinski's rule of five, removing compounds that did not meet the bioavailability criteria, resulting in 285 active molecules. Next, we predicted the potential targets of these active compounds through PubChem and ChEMBL databases (Fig. 5A), and the final total number of target-containing compounds was 141. In the PubChem and ChEMBL databases, we identified 491 shared target genes (Fig. 5B). To further understand the relationship between these active molecules and target genes (Fig. 5C), we statistically analysed the binding of different compounds to their target genes. The results showed that quercetin had the highest number of bindings to target genes at nearly 3,000, and other compounds such as coumarin, genistein and ethanol were also associated with a large number of target genes. To further focus on mechanisms associated with epigenetics, we screened target proteins associated with DNA methylation, particularly those associated with DNMTs. On this basis, we constructed a regulatory network of herbal compound-active molecule-DNA methylase protein (Fig. 5D). Figure 5E shows the overall fold of human DNMT1 (AlphaFold model; for structural context only). Independent docking to the C-terminal catalytic domain predicted that quercetin and

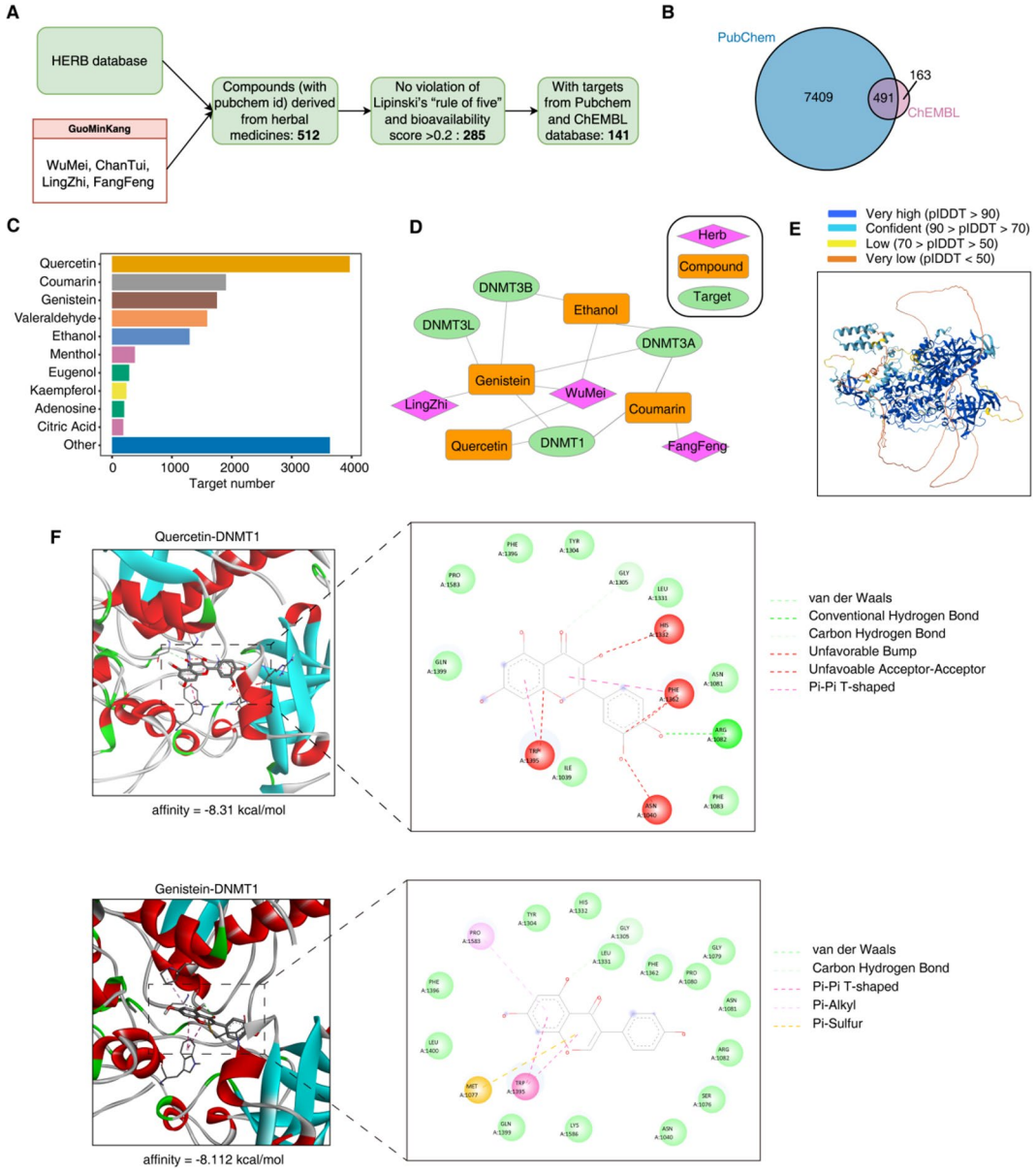

Fig. 5. Identification and prediction of active compounds of GMK and their DNA methyltransferase targets. (**A**) Workflow of compound selection from GMK. (**B**) Venn diagram of target overlap between PubChem and ChEMBL. (**C**) Bar graph showing the number of target genes associated with each active compound in GMK. (**D**) Network diagram of active molecules and herbal components constructed to target DNA methyltransferases. (**E**) Predicted protein structures of DNMT1 analyzed with AlphaFold3 algorithm, with high-ranking scores of 0.75. (**F**) Molecular docking of Quercetin and Genistein with DNMT1. Top: Quercetin–DNMT1; bottom: Genistein–DNMT1. Left panels show 3D binding poses; right panels depict 2D interaction maps with key residues (chain A numbering). Ligands are shown as sticks and colored by atom type: C (dark gray/black), O (red), N (blue), H (white), S (yellow). DNMT1 is rendered as a cartoon colored by secondary structure: α-helix (red), β-sheet (cyan), turns/bends (green), coils/loops (gray). Interaction legend (right panels): van der Waals (green dotted), conventional H-bond (light-green dashed), C–H bond (dark-green dashed), π–π T-shaped (pink dashed), π–alkyl (purple dashed), π–sulfur (yellow dashed), unfavorable contacts including bump or acceptor–acceptor clash (red dashed).

genistein bind in the same pocket, which geometrically overlaps the canonical SAM cofactor–binding cleft. Both ligands adopt highly overlapping poses stabilized mainly by π–π/π-alkyl contacts and hydrogen bonds, with predicted binding free energies of − 8.31 kcal/mol (quercetin) and − 8.112 kcal/mol (genistein) (Fig. 5F). The network revealed that key compounds such as quercetin, coumarin, and genistein in herbs such as umeboshi, ganoderma lucidum, and fengfeng may exert epigenetic regulation by targeting the protein activity of DNA methyltransferases (e.g., DNMT3A, DNMT3B, DNMT3L etc.) (Figure S2).Through this regulatory network, we

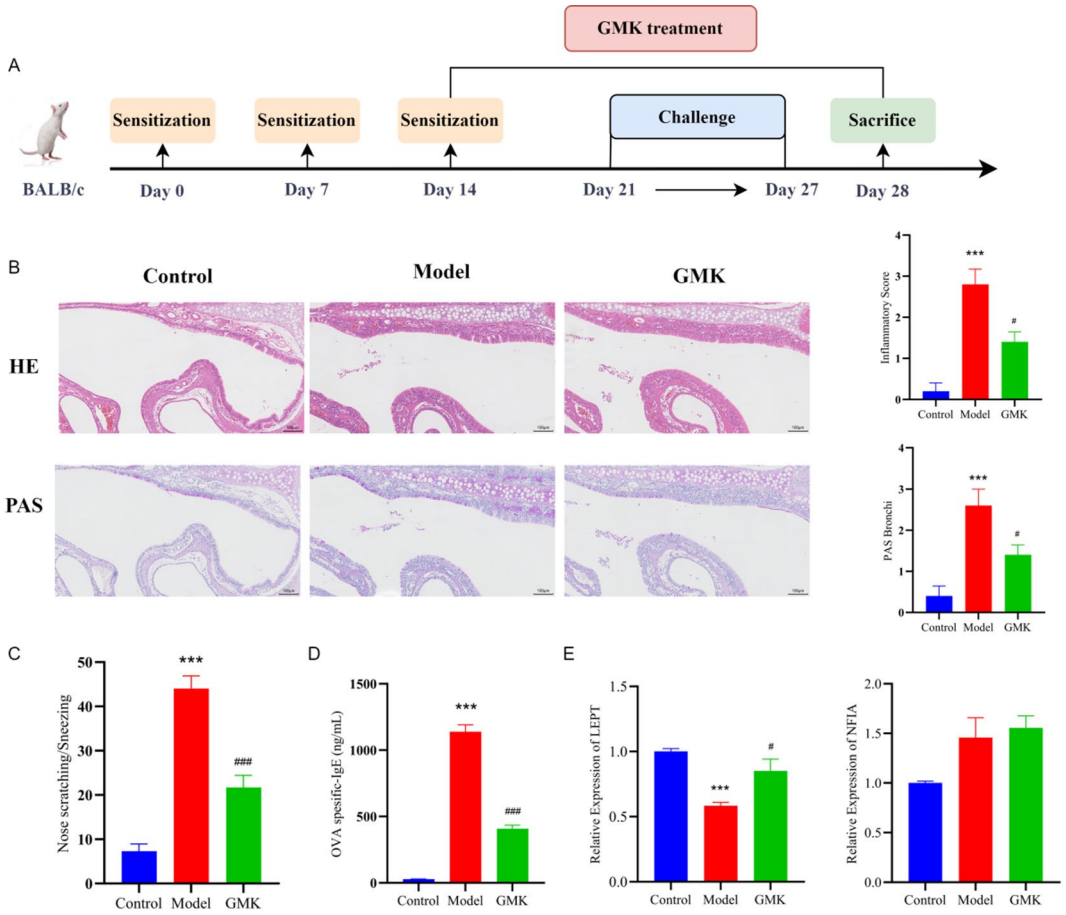

Fig. 6. Establishment of the AR mouse model and assessment of the effect of GMK on its treatment. (**A**) Schematic diagram of the experimental design. (**B**) Histopathological analysis of nasal mucosa. (**C**) Nasal scratching/sneezing behaviour scores. (**D**) OVA-specific IgE levels in serum. (**E**) Relative expression levels of LEPT and NFIA in nasal mucosal tissues. Note: Data are expressed as mean ± standard deviation and statistical differences were determined by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. $^{***}p < 0.001$, $^{*}p < 0.05$, $^{###}P < 0.01$, $^{#}P < 0.05$.

not only identified the possible biological targets of TCM compounding, but also provided a theoretical basis for its intervention in AR through epigenetic mechanisms.

Therapeutic effect of GMK on mouse model of AR

The mouse model of AR was constructed by OVA method (Fig. 6A). HE, PAS, and staining analyses (Fig. 6B) showed that there was obvious glandular hyperplasia and a significant increase in the number and volume of the glands in the nasal mucous membrane tissue of the mice in the model group. In the GMK group, the proliferation of glands in the nasal mucosa tissue was significantly alleviated, and the number and volume of glands were reduced. From the behavioural results of the mice, it can be seen that GMK can reduce the number of nose scratching and sneezing, and effectively alleviate the symptoms of AR. Compared with the control group, the model group exhibited significantly higher rhinitis symptoms ($P < 0.01$), and compared with the model group the GMK group showed lower symptoms ($P < 0.01$), indicating that GMK had a significant alleviating effect on the symptoms of AR (Fig. 6C). ELISA results showed that the total IgE level in the serum of mice in the model group was significantly higher than that in the blank control group ($P < 0.001$). The total IgE level in the serum of mice was significantly reduced after GMK treatment ($P < 0.001$), suggesting that GMK may alleviate allergy symptoms by regulating the immune response and inhibiting the synthesis and release of IgE (Fig. 6D). RT-qPCR results showed that the expression of *LEPR* in the nasal mucosa of mice in the model group was reduced ($P < 0.001$), and the expression increased significantly after GMK treatment ($P < 0.01$), and the expression of NFIA did not change significantly before and after GMK treatment, which was not statistically significant. It suggests that GMK may affect the expression of *LEPR*-related genes by regulating the level of DNA methylation, which in turn suppresses inflammatory response and abnormal immune cell function (Fig. 6E).

Discussion

The aim of this study was to investigate the epigenetic regulatory mechanism of GMK in the treatment of AR. By analysing the DNA methylation patterns and gene expression profiles of the healthy control group, the group

of untreated AR patients, and the group of AR patients treated with GMK, combined with the analysis of the transcriptome data, the changes in the proportion of immune cells, the analysis of the network pharmacology, and the experiments on animal models, we revealed that GMK may regulate DNA methylation by modulating the pattern, restoring the aberrant methylation status in AR patients, which in turn affects the expression of key genes, regulates the proportion of immune cells, and ultimately alleviates AR symptoms.

AR as a common chronic inflammatory disease, has a complex pathogenesis involving abnormal immune system responses and epigenetic changes. In recent years, DNA methylation, a key epigenetic mechanism, has been shown to play an important role in the onset and development of AR[31,32]. The results showed a significant increase in the number of DMRs, especially Hypo-DMRs, after GMK treatment, suggesting that GMK has a strong regulatory effect on DNA methylation patterns. In addition, the GO enrichment analysis and KEGG enrichment analysis showed that the genes with altered methylation levels after GMK treatment were involved in key biological processes such as immune regulation, epithelial cell function, and metabolic pathways, suggesting that GMK may alleviate the symptoms of AR by regulating the methylation status of these key genes and restoring the balance of the immune system and epithelial barrier function. The structure of the nasal mucosal epithelial barrier consists of tight junctions, adherens junctions, bridges and hemibridges. Tight junctions are disrupted and the nasal epithelial barrier is impaired in patients with AR[33]. It has been shown that enhancing nasal mucosal epithelial cell proliferation and inhibiting apoptosis, inflammation and barrier damage can alleviate AR symptoms. Studies have shown a strong correlation between altered DNA methylation in the nasal mucosal epithelium and IgE sensitisation[34]. Total immunoglobulin E (tIgE) levels were significantly reduced in patients after GMK treatment, suggesting that it may play an important role in immunomodulation. The central role of IgE in allergic diseases, including allergic rhinitis, and the importance of its mediated immune response in disease onset and progression[32]. It is suggested that GMK may restore aberrant methylation patterns in AR patients by regulating specific DNA methylation regions, thereby improving the immune homeostasis and epithelial barrier function of patients and alleviating AR symptoms.

Analysis of PBMC transcriptome sequencing data from AR patients revealed that the gene expression profiles and immune cell ratios were significantly different from those of healthy controls. Significantly up-regulated DEGs in AR patients were mainly involved in the regulation of viral entry into the host cell, regulation of bioprocesses of symbiotic interactions, nucleosome assembly, and the regulation of innate immune responses; while significantly down-regulated DEGs were mainly associated with taste perception, cell polarity establishment and Wnt signalling pathway. In addition, GSEA results showed that the expression levels of gene sets related to allograft rejection, inflammatory response and *IFN-α* response were significantly increased in the AR group, which further proved that the occurrence of AR was closely related to the abnormal response of the immune system. Analysis of immune cell ratios showed a significant decrease in the proportion of CD8 T cells and a significant increase in the proportion of neutrophils in patients with AR, suggesting that an imbalance in immune cell ratios may lead to over-activation of allergic reactions and exacerbation of inflammatory reactions, which can lead to itchy, stuffy, runny nose and other symptoms. These results provide an important basis for an in-depth understanding of the pathogenesis of AR.

To further explore the mechanism by which GMK regulates gene expression through methylation, we performed an overlap analysis between DMGs recovered to normal after GMK treatment and DEGs in the AR disease group, and screened 10 overlapping genes, including *ESF1*, *ZRANB2*, *DCLK2*, *PEG10*, *DUSP19*, *LEPR*, *DYNC2H1*, *DAB2IP*, *NFIA* and *CTD-2260A17.2*. The expression of these genes differed significantly between the AR group and the healthy group, and the expression levels of the remaining nine genes, except *CTD-2260A17.2*, were significantly positively correlated with the proportion of CD8 T cells and significantly negatively correlated with the proportion of neutrophils. This finding is consistent with a significant decrease in the proportion of CD8 T cells and a significant increase in the proportion of neutrophils in AR patients[35], further supporting the potential role of these overlapping genes in GMK treatment of AR. The immune system occupies a central position in the pathogenesis of AR, which is manifested by the imbalance of Th1/Th2 responses and the abnormal function of multiple immune cells[36]. By modulating the methylation of relevant genes, GMK may influence T-cell differentiation and function, restore immune balance and reduce inflammatory responses. It is suggested that GMK may play a role in the treatment of AR by modulating the methylation status of these genes, which in turn affects their expression and thus regulates immune cell ratios. It is important to note that DNA methylation is a dynamic process maintained by the balanced action of methyltransferases (DNMTs) and demethylases. The Ten-Eleven Translocation (TET) family of enzymes, which catalyze the oxidation of 5-methylcytosine (5mC) to initiate active demethylation, play a crucial role in this balance[37]. While our current findings highlight a potential role for DNMTs, the contribution of TET protein activity to the epigenetic restoration observed in GMK-treated groups remains an open and compelling question for future investigation.

In addition, through web-based pharmacological analyses, we predicted the active ingredients in GMK and their potential target genes, especially those related to DNMTs. We constructed a regulatory network of herbal compound-active molecule-DNA methylation enzyme genes and revealed that key compounds such as quercetin, coumarin, and genistein in the herbs may play an epigenetic regulatory role by regulating DNA methyltransferase genes (e.g., DNMT1, DNMT3A, DNMT3B, etc.) to exert epigenetic regulation. Molecular docking analyses revealed that the active molecules in GMK, Quercetin (affinity = −8.31 kcal/mol) and Genistein (affinity = −8.112 kcal/mol), have a high affinity for DNMT1. These results suggest that the active components in GMK may regulate the methylation status of genes through interaction with DNMTs, thereby affecting gene expression and immune cell function. In addition, compounds such as quercetin, coumarin and gerberellin have been shown in other studies to modulate the immune system and inflammatory response. For example, quercetin has been shown to improve Th1/Th2 cell imbalance and reduce symptoms of AR[36,38,39]. These findings further support the mechanism by which GMK exerts epigenetic regulation through its active components.

The experimental results of the AR mouse model constructed by OVA method showed that GMK could reduce the number of nose scratching and sneezing in mice and effectively alleviate the symptoms of rhinitis. Histological analysis showed that glandular hyperplasia was alleviated in the nasal mucosa tissue of GMK-treated mice, and the number and volume of glands were reduced. In addition, the level of cytokine IgE in the nasal mucosa of GMK-treated mice was significantly reduced, and the expression level of methylase-related genes was also significantly changed. In the immune system, the regulatory role of leptin involves processes such as CD 4 + T cell differentiation[40]. *LEPR* signalling can influence immune cell function and inflammatory responses, e.g., in microglia, the absence of *LEPR* leads to a cellular progression in a pro-inflammatory direction manifested by an increase in the expression of inflammatory factors (e.g., *IL-6*, *TNF-α*, etc.). Maintenance of *LEPR* signalling in alveolar macrophages attenuates lung inflammation. *LEPR* signalling protects macrophages from necrotic apoptosis, thereby limiting neutrophil recruitment and pro-inflammatory cytokine release[41]. *NFIA* in adipocytes down-regulates pro-inflammatory cytokines to ameliorate adipose tissue inflammation[42]. *NFIA* exerts region-specific and injury-specific effects in reactive astrocytes, acting as either pro- or anti-inflammatory. In this study, qPCR was used to preliminarily verify the expression of differential genes in mice, and the increased expression of *LEPR* after GMK treatment may be due to the role played by the reduction of its methylation level through the regulation of DNMTs after the action of GMK. *NFIA* showed no significant change before and after drug administration, and the expression trend in mice was not consistent with the trend of human results, which might be due to the difference in species, and the sample size could be increased for further study. These results further confirmed that GMK plays a role in the treatment of AR by regulating DNA methylation levels and inhibiting the expression of related genes.

Limitations of the study

Although this study has preliminarily elucidated the epigenetic regulatory mechanisms of GMK in treating AR through analysis of DNA methylation and transcriptomic data, network pharmacology analysis, and animal experiments, certain limitations exist. Firstly, the sample size is relatively small, necessitating further expansion to validate the reliability of the findings. Secondly, network pharmacology analysis and molecular docking simulations rely on existing databases (such as PubChem and ChEMBL) and computational algorithms. Their accuracy depends on the quality and completeness of these databases, as well as the precision of the algorithms, which may influence the identification of active compounds and their target genes. Thirdly, although AlphaFold demonstrates outstanding performance in protein structure prediction, discrepancies may exist between predicted structures and actual functional sites within proteins. Further experimental validation is required to confirm the functional relevance between predicted compounds and target proteins. Fourthly, the omics technologies employed in this study, including DNA methylation and transcriptome sequencing, primarily revealed associations between genetic and epigenetic alterations and disease states. The identified DMRs and DEGs may be associated with AR, but their causal role in AR pathogenesis requires further functional validation. Future research should focus on elucidating the mechanism of GMK in AR treatment by expanding sample sizes, conducting rigorous experimental validation, and applying advanced functional genomics techniques.

Conclusion

In this study, we revealed the epigenetic regulatory mechanism of GMK in the treatment of AR by integrating DNA methylation and transcriptome sequencing data. The findings suggest that GMK is able to restore the aberrant methylation pattern in AR patients by regulating specific DNA methylation regions, which in turn affects the expression of relevant genes and the proportion of immune cells, and ultimately alleviates AR symptoms.

Data availability

The data and materials in this study are available from the corresponding author upon request.

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Author contributions

Designed the experiments: QW and JW. Performed the experiments: YMZ LMY HYZ JTL HLZ LHH. Analyzed the data: LMY HYZ. Manuscript preparation: LMY. Study supervision: JW. All authors were involved in the formulation of the research questions and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Informed consent

This use of the database does not require the consent of the owner. The databases on humans used in this paper are publicly available and allow for unrestricted re-use through an open licence.

Consent for publication

Not applicable.

Additional information

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