



Mrgprb2 gene plays a role in the anaphylactoid reactions induced by *Houttuynia cordata* injection

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ABSTRACT

Ethnopharmacological relevance: *Houttuynia cordata* Thunb., a plant belonging to the family of Saururaceae, has been used as a traditional Chinese medicine for more than 1500 years. Because of its various pharmacological activities, it was widely used as antipyretic, detoxification, anti-inflammatory drugs. *Houttuynia cordata* (HC) injection was prepared using contemporary methods to extract effective components from *H. cordata* Thunb. However, the adverse event reports of HC injection are accumulating remarkably with the HC injection clinical applications increased. Previous studies demonstrated that the major side effects of HC injection were anaphylactoid reactions. Our work might shed the light on the role of Mas-related G-protein coupled receptor-X2 (MRGPRX2) in modulating drug-induced anaphylactoid reactions.

Aim of the study: We aimed to investigate the role of the mouse Mas-related G-protein coupled receptor B2 (*Mrgprb2*) (the orthologous gene of human MRGPRX2) in anaphylactoid reactions induced by HC injection.

Materials and methods: *Mrgprb2* related anaphylactoid reactions induced by HC injection were investigated by histamine/ β -hexosaminidase releasing, mast cell degranulation, and hind paw swelling assays by using a *Mrgprb2* knockout mouse model. Furthermore, the transcriptomic profiles of the anaphylactoid reaction induced by HC injection was analyzed by RNA sequencing.

Results: Mice without *Mrgprb2* exhibited significantly decreasing in mast cell degranulation, serum histamine release, and hind paw swelling degrees. The RNA sequencing results indicated that *Mrgprb2* could play a pivotal role in HC injection induced anaphylactoid reaction mediated by mTOR/AMPK pathway. Intriguingly, our results showed that *Mrgprb2* might involve in Compound 48/80 induced anaphylactoid reactions mediated by Reelin/E-cadherin axis, which suggested different roles of *Mrgprb2* in anaphylactoid reactions induced by HC injection and C48/80.

Conclusion: Our studies reported effects and underlying mechanisms of *Mrgprb2* in the anaphylactoid reaction induced by HC injection.

1. Introduction

Houttuynia cordata (HC) injection contains extracts from *H. cordata* Thunb. Its main components include volatile compounds, flavonoids, phenolic acids, and compounds containing organic nitrogen (Chiu et al., 2016; Yang et al., 2016). This injection was clinically used for 50 years in China because of its broad pharmacological activities, include antibacterial, anti-inflammatory, immunoregulatory, and specific antiviral effects (Li, 2014). *Houttuynia cordata* injection is administered intravenously (i.v.) to treat mainly respiratory, digestive, ear, nose,

throat, oral cavity, and skin infections (Cao, 2016). However, emerging evidence suggests that HC injections can cause several adverse reactions such as dyspnea, pruritus, cheek swelling, rash, anaphylactic shock, and cardiac arrest (Wei, 2010). Follow-up studies showed that HC intravenous injection (i.v.) can induce type I allergy (allergic reaction) and anaphylactoid reaction (Liu et al., 2010). And the latter one was the main cause of significant growth of serious adverse event reports in clinic (Liang et al., 2015). What is more, serious adverse events have already resulted in seven types of HC injections banned by the State Food and Drug Administration in July 2006 in China, however, the

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resting 89 types of HC injections are still in use.

An anaphylactoid reaction is a dose-dependent allergic reaction without IgE mediation that usually appears after the first dosage (Fan et al., 2018; Li et al., 2019). Anaphylactoid reactions can be caused by substances that directly activate allergic inflammatory effector cells or mast cells (Liu et al., 2020). Tatemoto et al. found that the human mast cells induced anaphylactoid reaction via Mas-related G-protein coupled receptor-X2 (MRGPRX2) (Tatemoto et al., 2018). Basic secretions such as Compound (C) 48/80 and Substance P stimulate mast cells through MRGPRX2 receptors, resulting in cell degranulation and elevated intracellular calcium concentrations. And McNeil et al. demonstrated that Mrprgb2 receptors (mouse orthologue of human MRGPRX2) could be activated by basic secretions and small molecule drugs (McNeil et al., 2015). Additionally, some traditional Chinese medicine injections (TCMIs) might activate mast cells via MRGPRX2 in drug-induced anaphylactoid reactions, such as Sinomenine hydrochloride (Liu et al., 2017) and Kudiezi injection (Lin et al., 2018). However, the detailed role of MRGPRX2 in the anaphylactoid reaction induced by HC injection is still unclear. Therefore, it is necessary to elucidate the mechanism of the anaphylactoid reactions induced by HC injection.

In this work, *Mrgprb2* (the orthologue of human MRGPRX2) knockout mice were generated by CRISPR/Cas9 technology. Our current study investigated the role and underlying mechanism of *Mrgprb2* in the anaphylactoid reaction induced by HC injection. Our work might provide a better understanding of the mechanisms involved in traditional Chinese herbal medicine induced anaphylactoid reactions and possible target to improve their clinical applications.

2. Materials and methods

2.1. Animals

Mrgprb2 knockout (KO) mouse models were constructed (Gem-Pharmatech Co., Ltd., Nanjing, Jiangsu, China). Wild-type C57BL/6 mice (Beijing Vitong Lihua Experimental Animal Technology Co., Ltd., Beijing, China) aged 6–8 weeks were used. Animals were housed at the Experimental Animal Center of Jiangsu University (Zhenjiang, Jiangsu, China) and were provided with food and water ad libitum. The mice were reared and bred in a room at 26 °C under 40% humidity at the Experimental Animal Center of Jiangsu University. All animals were treated equally regardless of the experimental protocol.

2.2. Ethics statement

The study was conducted in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols involving the mice were approved by the Animal Ethics Committee at Jiangsu University, Zhenjiang, China (Permit Number: U-I-2019110703).

2.3. Drugs and reagents

HC injection was obtained from Furen Pharmaceutical Group Co., Ltd. (Lot No.: Z20073101, 2 mL, Zhoukou, Henan, China). β -Hexosamine and Compound 48/80 (C48/80) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Saline was purchased from Anhui Shuanghe Pharmaceutical Co., Ltd. (Wuhu, Anhui, China). Evans Blue was obtained from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan). The Animal Genomic DNA Rapid Extraction Kit was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The mouse histamine ELISA kit was obtained from Zhenke Biological Technology Co., Ltd. (Shanghai, China). The Qubit RNA Detection Kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). HiSeq NGS® MaxUp II Dual-mode mRNA Library Prep Kit for Illumina® and HiSeq NGS® DNA Selection Beads were obtained from Yeasen Biotechnology

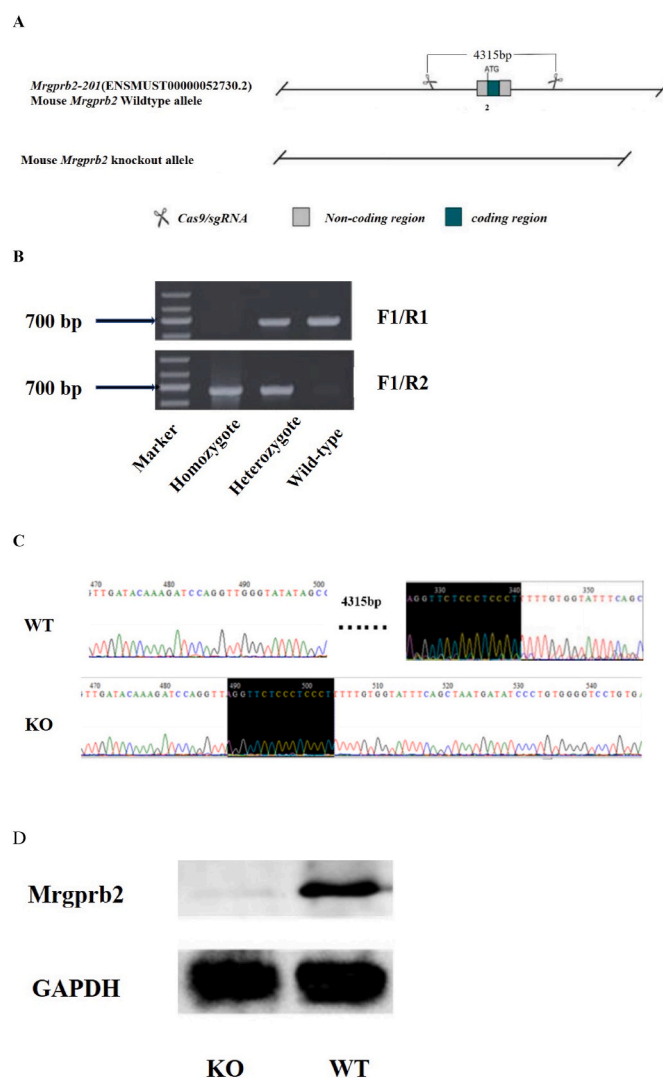


Fig. 1. Mouse model generation. (A) Gene knockout strategy. (B) Genotype identification and target bands at 700 bp. (C) Sequencing results showing two mouse genes that differ by 480 bases. (D) Protein expression of MRGPRX2 in mast cells from two types of mice.

Co., Ltd. (Shanghai, China).

2.4. Mice model construction assay

We edited *Mrgprb2* using CRISPR/cas9 (Fig. 1A). According to the structure of *Mrgprb2*, exon2 of the *Mrgprb2-201* transcript (ENSMUST0000052730.2) was recommended to be the knockout region, as it has the start codon, ATG. Protein functions were disrupted by knocking out this region. Single-guide RNA (sgRNA) was transcribed in vitro. We microinjected Cas9 and sgRNA into the fertilized eggs from C57BL/6J mice, which were transplanted to obtain model *Mrgprb2*^{+/-} F₀ mice, as confirmed by PCR and sequencing. The sequences of the sgRNAs were as follows: sgRNA1: ATCAGGCTATATACCAACC; sgRNA2: CCTCTGGATATGGTCATGTT. We then created homozygous KO mice by propagating *Mrgprb2*^{+/-} F₀ mice at a male: female ratio of 1:1 to obtain the F₁ mice.

2.5. Mouse model gene identification

The toes of 7-10-day-old mice were cut with sterile surgical scissors and lysed using the animal genomic DNA rapid extraction kit.

Primers were designed using Primer Premier 5.0. The extracted DNA

was used as the template for PCR amplification. The primer sequences were as follows: WT mice, forward (F1), 5'-TCCTTGATTGGACTGCTACT-3', and reverse (R1), 5'-CAGTTCTGCGTAAATGTCTT-3'; KO mice, forward (F1), 5'-TCCTTGATTGGACTGCTACT-3', and reverse (R2), 5'-GTGTAGTAATGGGAGATGGAG-3'. Target bands resolved at 700 bp. The PCR reaction mix contained 12.5 μ L of 2 $\times$ Taq master mix (Sangon Biotech, Shanghai, China), 9.5 μ L of double distilled water, and 1 μ L each of sample DNA and forward and reverse primers. The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 35 s, followed by a final extension reaction at 72 °C for 10 min. The target gene was amplified using an Applied Biosystems 2720 thermal cycler (Thermo Fisher Scientific Inc.). The PCR products were resolved by electrophoresis on 1% agarose gels, then visualized using an LUV-260 UV lamp (Luyor Corporation, Shanghai, China). The PCR products with clearly distinguishable genotypes were selected and sequenced (Shanghai Sangon Biotech) using the same primers that were used for PCR amplification.

2.6. Western blotting

Ten KO and ten WT mice were euthanized by CO₂ inhalation. Peritoneal mast cells were extracted using sequential peritoneal lavage. After removing the red blood cells, the cells were lysed with RIPA buffer containing phenyl-methyl sulfonyl fluoride at 4 °C for 30 min. Insoluble protein from the cell lysate was removed by centrifugation at 13,800 $\times$ g for 10 min at 4 °C. The protein concentration in the supernatant was determined using Enhanced BCA Protein Assay Kits (Beyotime Biotechnology). Sodium dodecyl sulfate protein loading buffer (5 $\times$) was added to the supernatant, which was then boiled for 5 min and stored at -80 °C. Proteins (10 μ g) were resolved by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membranes were placed in an ice bath for 1 h, then non-specific antibody binding was blocked in 5% skimmed milk for 1 h at 37 °C. After three washes (10 min each) with Tris-buffered saline containing 0.2% Tween-20 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), the membranes were incubated with the primary anti-MRGX2 antibody (1:1000; Novus Biologicals, Littleton, Centennial, CO, USA) at 37 °C for 2 h. After three washes (10 min each) with Tris-buffered saline containing 0.2% Tween-20, the membranes were incubated with an anti-rabbit IgG secondary antibody (1:1000; Beyotime Biotechnology Co., Ltd., Shanghai, China) at 37 °C for 1 h. The membranes were incubated with ECL detection reagents (Beyotime Biotechnology Co., Ltd.), then protein levels were evaluated using a chemiluminescent imager. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Proteintech, Rosemont, IL, USA) was used as reference for normalization of the protein expression levels.

2.7. Peritoneal mast cell extraction

Ten KO and ten WT mice were euthanized by CO₂ inhalation, and peritoneal mast cells were extracted by sequential peritoneal lavages. Red blood cells were removed using ACK lysis buffer (Beyotime Biotechnology Co., Ltd.). The cells were resuspended in Tyrode salt solution (Beijing Cool Laibo Technology Co., Ltd., Beijing, China), then mast cells were obtained by Percoll discontinuous density gradient centrifugation at 200 $\times$ g for 5 min at 4 °C. The cells were collected from the interface and washed twice with ice-cold phosphate-buffered saline (PBS). Trypan blue staining and morphological analysis confirmed that the purity of the mast cells was >95%. The extracted mast cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.).

2.8. β -hexosaminidase assay

For this assay, the culture medium was removed, and the cells were resuspended in Tyrode salt solution, and incubated with various concentrations of HC injection or C48/80. To analyze the total β -hexosaminidase content, cells were lysed with 0.1% Triton X-100 in Tyrode salt solution. The supernatant was removed after centrifugation, then the cells (2 $\times$ 10⁵/well) were seeded into 48-well plates containing 300 μ L of citric acid/sodium citrate buffer (Leagene Biotechnology, Beijing, China) and incubated for 1 h at 37 °C in 5% CO₂ atmosphere. Thereafter, 30 μ L of the supernatant from each group was incubated with β -hexosamine solution (Sigma Aldrich Corp.) in 96-well plates for 2 h. Sodium carbonate/sodium bicarbonate buffer solution (Leagene Biotechnology) was added to stop the reaction, following which, the concentration of β -hexosaminidase released from the cells was assessed by determining the absorbance of the samples at 410 nm using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The β -hexosaminidase released was calculated using the following formula: β -hexosaminidase release rate (%) = (OD of sample cell - OD of sample cell treated without drugs)/(OD of sample cell lysed by 0.1% Triton X-100 - OD of sample cell treated without drugs) $\times$ 100%.

2.9. Cell morphological assay

We discarded the medium from the extracted mast cells and washed them three times with PBS. Then, the cells were incubated for 1 h and centrifuged at 200 $\times$ g at 4 °C for 15 min. The precipitated cells were fixed in 2.5% glutaraldehyde, and then frozen and cut into ultra-thin sections. Morphological changes in mast cell degranulation were assessed using an ECLIPSE 100-F transmission electron microscope (Nikon, Tokyo, Japan).

2.10. Histamine release assay

Forty WT mice and forty KO mice were injected intravenously (i.v.) with different concentration of drugs at 20 mL/kg (Yi et al., 2015). Blood collected from the venous sinuses of the mice at 30 min after administration was stored at 20 °C for 1–2 h, and then centrifuged at 1200 $\times$ g for 10 min. Serum was decanted and stored at -80 °C. Histamine release from the serum was measured using mouse histamine ELISA kits.

2.11. Hind paw swelling and histochemistry

Twelve WT mice and twelve KO mice were administered (i.v.) with different concentration of drugs at 20 mL/kg, which was followed (after 5 min) by an i.v. injection with Evans blue (20 mL/kg). The mice were sacrificed by decapitation 30 min later, and their hind paws were photographed.

The ears and lung lobes of the mice were collected, postfixed in 10% formaldehyde for 14 d, embedded in paraffin, and sectioned for hematoxylin-eosin (HE) staining. The morphology of the tissue sections was assessed by transmission electron microscopy.

2.12. Transcriptomic profile analysis

2.12.1. RNA sequencing

Twelve KO mice and twelve WT mice were administered with different concentrations of saline, C48/80 or HC injection, and then were euthanized via CO₂ inhalation after 30 min of drug administration. The mast cells were extracted and stored at -80 °C.

mRNA was separated from total RNA obtained from the mast cells using Qubit RNA detection kits, and reverse-transcribed into double-stranded cDNA, which was purified, quantified and amplified using Hieff NGS® DNA Selection Beads and agarose gel. The quality check was performed using Hieff NGS® MaxUp II Dual-mode mRNA Library Prep Kit for Illumina® to obtain a cDNA library that met the requirements of

Illumina sequencing.

2.12.2. GO and KEGG pathway analysis

The cDNA library was sequenced using an Illumina Novaseq 6000 (Inc., San Diego, CA, USA), and raw reads were obtained after CASAVA base calling. The quality of the raw data was evaluated using FastQC. We used Trimmomatic software for data filtering to obtain accurate and effective data. To get clean reads, reads with N bases, joint sequences in reads (forward: AGATCGGAAGAGCACACGTCTGAA; reverse: AGATCGGAAGAGCGTCGTGTAGGGA), low-quality bases ($Q < 20$), base with reads tail mass below 20, reads less than 35 nt in length and their paired reads were removed. Mus musculus GRCm38 (http://no.v2020.archive.ensembl.org/Mus_musculus/Info/Index) was used as the specific reference genomes, and HISAT2 was used to compare the sequences obtained after quality control (QC) with the reference genome. The results were statistically compared using RSeQC.

The samples with biological replicates were analyzed using DESeq, and the significantly differentially expressed genes (DEGs) with false discovery rate < 0.05 and a $|\text{fold change}| > 2$, and at least one expression (TPM) is greater than or equal to 5 in the two samples compared were selected.

We analyzed DEG enrichment using the GO (<http://geneontology.org>) and KEGG (<https://www.kegg.jp/>) databases to clarify differences in gene functions. DEGs were imported into GO database and KEGG database, respectively. The hypergeometric test was used to calculate the P value enriched to the pathway. The P value was corrected to obtain the q value, and the q value < 0.05 was used as the screening standard for the significant enrichment pathway.

2.13. Real-time quantitative PCR

Real-time quantitative PCR was performed on the Roche Light-Cycle®96 using the SYBR Green reagent (Beyotime Biotechnology). GAPDH was used as internal control. The PCR primers were listed as follows: GAPDH forwards: 5'-TGACAACCTTGGTATCGTGAAGG-3', and reverse: 5'-AGGCAGGGATGATGTTCTGGAGAG-3'; Sesn2 forwards: 5'-CCTTTGCCCTCATACCTTTTCTCC-3', and reverse: 5'-CACCAA-CAGCCACTCCTTACTCTTC-3'; Reln: 5'-TGACAACCTTGGTATCGTGAAGG-3', and reverse: 5'-GAAGACACGACTCCACAGCCTTG-3'; mTOR: 5'-ACCGTCGCCTTCACAGATACC-3', and reverse: 5'-GCAGTCCGTTCTTCTCCTTCTTG-3'; E-cadherin: 5'-ACCAGCAGTTCGTGTGTCGCAC-3', and reverse: 5'-GTTCTCGTTCTCCACTCTCAGATG-3'. The amplification conditions for Sesn2 and E-cadherin were as follows: 95 °C, 3 min; (95 °C, 10 s; 60 °C 30 s; 72 °C 30 s) for 40 cycles, and for Reln and mTOR were as follows: 95 °C, 3 min; (95 °C, 10 s; 55 °C 30 s) for 40 cycles. Relative gene expression was calculated by the $2^{-\Delta\Delta C_q}$ method. Each sample had three repeats.

2.14. Statistical analysis

All the experiments in this study were repeated at least three times, and group data are expressed as the means $\pm$ S.D. The normality of data was tested by the linear regression model of data. Independent sample variance analysis was used to determine the statistical significance of the comparisons, using SPSS software (SPSS, Inc. Chicago, IL, USA). Differences were considered as significant at $p < 0.05$. The graphing software was Excel (Microsoft, Seattle, WA, USA).

3. Results

3.1. Generations of Mrgprb2 knockout model mice

The genotypes, sequencing, and protein phenotypes of the F₁ generation were determined in 7-day-old mice (Fig. 1B). The primer pair F1/R2 was designed to specifically bind to the two flanks of the knockout sequence, and a reverse primer (R1) was designed to bind to a

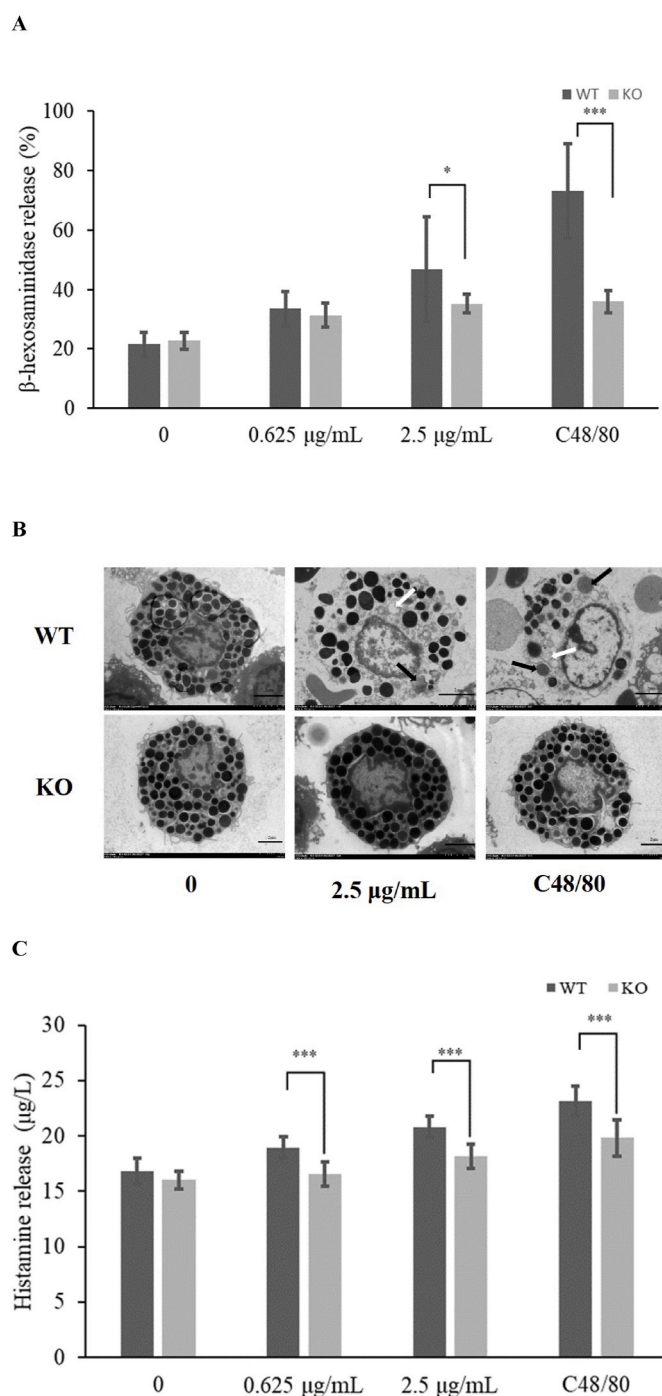


Fig. 2. *Houttuynia cordata* (HC) injection-induced mast cell degranulation. (A) β -hexosaminidase released by mast cells incubated with different concentrations of HC injection. (B) Morphological changes in mast cells incubated with different concentrations of HC injection. White and black arrows represent cavity structure and degranulation, respectively. (C) Histamine released by mast cells incubated with different concentrations of HC injection. Statistical significance was accepted at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

site inside the KO sequence. These two primer pairs generated products of approximately 700 bp in length. The PCR results identified WT, homozygous and heterozygous genotypes. The sequencing results were shown in Fig. 1C. Compared to the WT mice, the entire exon and parts of the intron in 3' UTR totaling 4315 bp were deleted in the KO mice, and the length was consistent with our gene knockout strategy.

The immunoblotting results were shown in Fig. 1D. Considering that

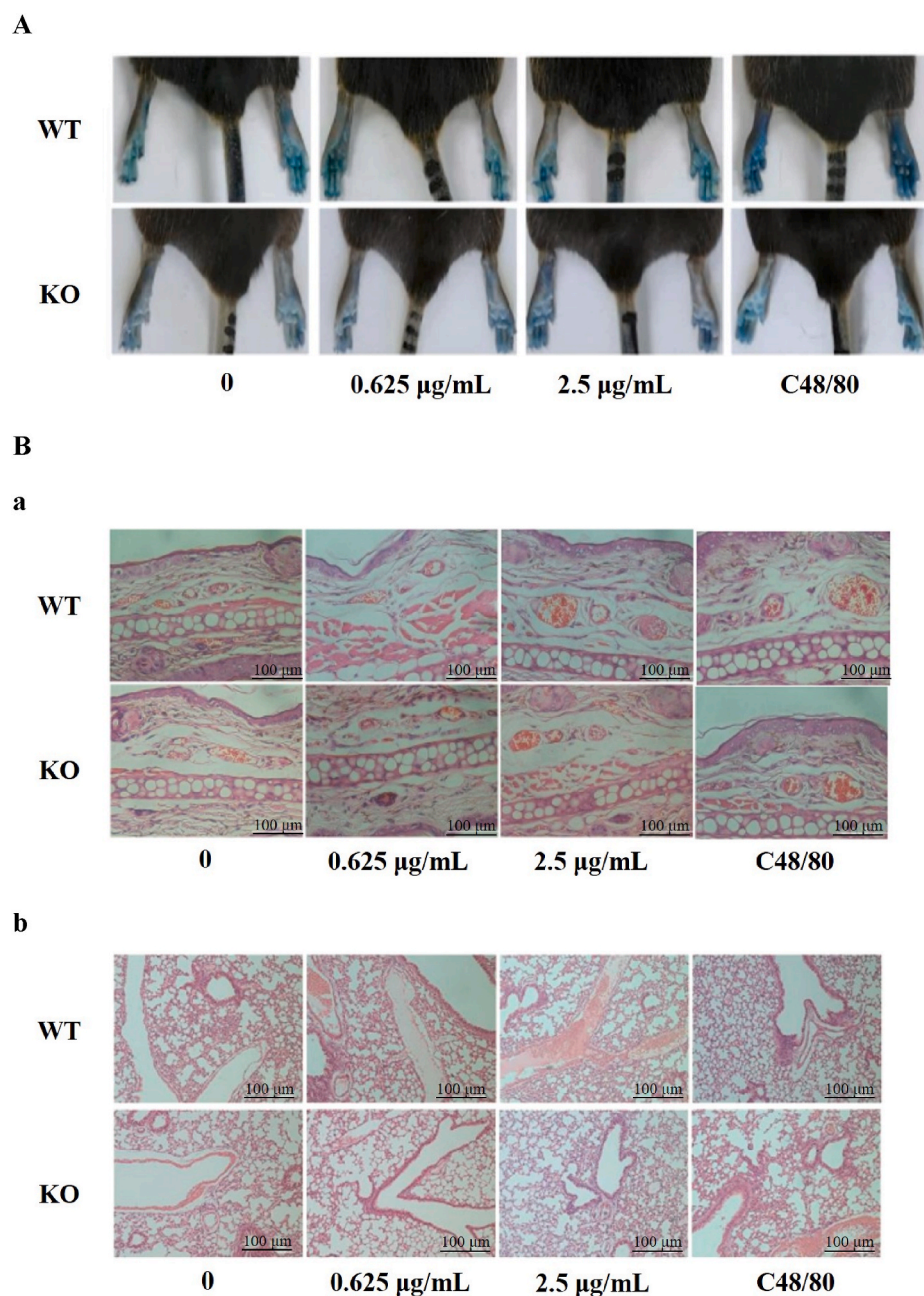


Fig. 3. *Houttuynia cordata* (HC) injection-induced anaphylactoid reactions in mice. (A) Blue staining and swelling of hind paws of mice induced by HC injection. (B) Pathological tissue sections of mouse (a) auricle and (b) lung (Scale bar: 100 µm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the antibody against Mrgprb2 was not commercially available, we used the anti-MRGPRX2 antibody for its specific recognition of both mouse and human orthologues. The results showed that Mrgprb2 was not expressed in KO mice, while Mrgprb2 expression was observed in WT mice.

3.2. Degranulation of mast cells was mediated by *Mrgprb2*

Anaphylactoid reactions are typically evaluated on the basis of mast cell degranulation and serum histamine release (Gao et al., 2017). We detected secreted β -hexosaminidase, which is a degranulation biomarker, augmenting histamine release. In our preliminary study, we found that the mild to moderate anaphylactoid reactions could be induced by the dosage of HC injection at 0.625–2.5 µg/mL. Therefore, in this study, mast cells were incubated with or without HC injection (0.625 or 2.5 µg/mL) for 1 h. The levels of secreted β -hexosaminidase were measured using 25 µg/mL C48/80 as the positive control.

According to Mouse Genome Informatics (MGI) data (<http://www.informatics.jax.org/>), mice homozygous for a KO allele have impaired mast cell activation and inflammatory responses to C48/80. Fig. 2A showed no significant difference between the amount of β -hexosaminidase released by mast cells from the mice injected with and without 0.625 µg/mL HC. Compared to WT mice, less amount of β -hexosaminidase was released in KO mice after 2.5 µg/mL HC injection, and the level of β -hexosaminidase release was significantly dropped after C48/80 injection. The normality of data was presented in Supplement Fig 1.

Fig. 2B showed the morphological changes in relation to the degranulation of peritoneal mast cells. The mast cells of KO and WT mice incubated with Tyrode salt solution were fully and evenly distributed, with distinct cell membranes. In contrast, peritoneal mast cells of the WT mice administered with HC injection and C48/80 had clear vacuoles, a decreased density of larger particles, an indistinct cell membrane boundary, particle membranes fused with the cell membrane, and particles scattered outside the cells. In summary, more degranulation was

Table 1

Comparison of sequenced samples and reference genomes.

	Raw reads	Clean reads	Total mapped	Multiple mapped	Uniquely mapped
NC1	62,955,020	59,772,416	55,316,148 (94.23%)	8,239,669 (14.04%)	47,076,479 (80.19%)
NC2	56,406,66	53,266,060	49,743,755 (94.53%)	7,145,077 (13.58%)	42,598,678 (80.95%)
NC3	57,340,048	54,243,414	50,190,186 (93.98%)	6,473,937 (12.12%)	43,716,249 (81.86%)
NC4	64,093,582	60,548,974	56,072,407 (94.13%)	6,425,911 (10.79%)	49,646,496 (83.34%)
NH1	74,551,606	71,519,104	66,001,456 (93.43%)	7,857,150 (11.12%)	58,144,306 (82.31%)
NH2	61,456,242	58,285,992	54,162,052 (94.03%)	8,110,504 (14.08%)	46,051,548 (79.95%)
NH3	59,543,866	56,519,126	52,557,286 (94.36%)	6,838,266 (12.28%)	45,719,020 (82.09%)
NH4	63,029,144	59,933,832	55,965,187 (94.10%)	8,008,619 (13.47%)	47,956,568 (80.64%)
N1	65,286,120	61,986,080	57,984,334 (94.10%)	8,382,430 (13.60%)	49,601,904 (80.50%)
N2	61,631,552	58,587,214	54,318,467 (94.04%)	6,641,678 (11.50%)	47,676,789 (82.55%)
N3	67,701,848	64,636,502	60,373,403 (94.33%)	7,614,406 (11.90%)	52,758,997 (82.43%)
N4	65,688,324	62,612,506	58,225,860 (93.78%)	9,182,041 (14.79%)	49,043,819 (78.99%)
KC1	63,516,832	60,208,398	55,718,708 (94.23%)	6,073,219 (10.27%)	49,645,489 (83.96%)
KC2	60,418,952	57,146,580	53,400,574 (94.41%)	6,316,748 (11.17%)	47,083,826 (83.24%)
KC3	62,400,500	59,169,596	54,692,758 (94.08%)	6,370,486 (10.96%)	48,322,272 (83.12%)
KC4	61,751,348	58,488,236	54,454,271 (94.25%)	7,156,415 (12.39%)	47,297,856 (81.86%)
KH1	62,142,332	58,981,378	55,385,038 (94.31%)	6,853,619 (11.67%)	48,531,419 (82.64%)
KH2	47,393,852	45,730,238	43,421,141 (95.79%)	7,202,874 (15.89%)	36,218,267 (79.90%)
KH3	49,116,372	47,318,656	45,069,853 (95.86%)	7,069,126 (15.04%)	38,000,727 (80.83%)
KH4	48,647,904	47,004,296	44,442,413 (95.57%)	7,282,502 (15.66%)	37,159,911 (79.91%)
K1	59,252,376	56,391,594	52,393,092 (94.54%)	9,275,218 (16.74%)	43,117,874 (77.81%)
K2	64,619,706	61,295,586	57,306,791 (94.03%)	7,733,263 (12.69%)	49,573,528 (81.34%)
K3	61,860,612	58,584,206	54,834,299 (93.90%)	6,842,889 (11.72%)	47,991,410 (82.18%)
K4	61,657,608	58,854,538	54,996,919 (94.55%)	5,619,956 (9.66%)	49,376,963 (84.89%)

evident in mice administered with C48/80 than in mice administered with HC injection. Furthermore, the morphology of peritoneal mast cells from KO mice exhibited no significant alterations, except for slightly enlarged size.

Fig. 2C showed that serum histamine release gradually increased with increasing concentrations of HC injection in WT mice. At high concentrations, the release was equivalent to that induced by C48/80. Serum histamine release slightly decreased in KO mice compared to that in WT mice. The normality of data was presented in Supplement Fig 2.

3.3. *Mrgprb2* played an important role in anaphylactoid reactions

The mice were administered with saline, or 0.625 or 2.5 $\mu\text{g/mL}$ of HC injection or C48/80, and then EB was injected (i.v.) into the mice to assess the hind paws thickness and blue staining. The degrees of blue staining in WT mice administered with saline and 0.625 $\mu\text{g/mL}$ HC injection were similar. The hind paws of WT mice administered with 2.5 $\mu\text{g/mL}$ of HC injection were stained darker blue and swollen. The degree of blue staining was the darkest in the hind paws of WT mice administered with C48/80. Compared with the degree in the WT mice, the degree of blue staining and swelling in the KO mice decreased. However, hind paw swelling remained significant in KO mice administered with C48/80 (Fig. 3A).

Fig. 3B shows the analyzed pathological tissue sections. Vasodilation was not evident in KO or WT mice administered with 0.625 $\mu\text{g/mL}$ of HC injection compared with those administered saline, whereas 2.5 $\mu\text{g/mL}$ HC injection significantly dilated auricle blood vessels in WT mice. The blood vessels of KO mice were also dilated, but to a lesser degree than those of the WT mice. Although C48/80 caused the most significant auricle vasodilation, vessels were less expanded in KO mice than in WT mice. Lung and auricle tissue sections were visually consistent. The degree of pulmonary inflammatory infiltration and pulmonary septal thickening was significantly lower in KO mice than in WT mice, and C48/80 did not elicit significant inflammatory symptoms.

3.4. Transcriptomic profile analysis

3.4.1. Data assembly and comparison

To gain insight into the role of *mrgprb2* in drug induced

anaphylactoid reactions, we performed genome-wide transcriptome profiling of mast cells collected from *mrgprb2*-deficient and wild type mice after treatment by RNA sequencing. A library was sequenced using the Illumina HiSeq™ sequencing platform. Samples meeting the requirements of database construction were sequenced.

The remove of splices and low-quality reads from the original sequencing data resulting in 45,730,238–71,519,104 high-quality clean reads, and the ratios (%) of Q30 and GC bases were 88.37%–90.85% and 47.79%–51.9%, respectively, indicating the richness of the sequencing data and that the quality was suitable for subsequent analysis. A comparison of the clean reads with the reference mouse genome, is shown in Table 1. The total mapped ratio was 93.43%–95.79%, with multiple and uniquely mapped reads accounted for 9.66%–16.74% and 79.90%–82.31%, respectively. The mice in the sequencing experiment were assigned to the following groups: N (n = 4), NH (n = 4), and NC (n = 4), WT mice administered with saline, HC injection, and C48/80, respectively, and K (n = 4), KH (n = 4), and KC (n = 4), KO mice administered with saline, HC injection, and C48/80, respectively. The NH and KH groups were experimental, and N, K, NC, KC were controls.

3.4.2. Screening and identification of DEGs

We identified 137 DEGs in the NH group, of which 49 (35.77%) and 88 (64.23%) were upregulated and downregulated, respectively, compared to the expression of the genes in the N group; in the NC group, we identified 206 DEGs, of which 112 (54.37%) and 94 (45.63%) were upregulated and downregulated, respectively. We also identified 75 DEGs in the KH group, of which 24 (32.00%) and 51 (68.00%) were upregulated and downregulated, respectively, compared to the expression of the genes in the K group; in the KC group, we identified 148 DEGs, of which 145 (97.97%) and 3 (2.03%) were upregulated and downregulated, respectively, (Fig. 4A).

3.4.3. GO analysis of DEGs

The massive GO database classifies molecular functions and describes the attributes of genes in organisms. Due to the huge amount of data, we selected only the top 30 most enriched data points to create a scatter plot of the significantly enriched functions of DEGs.

The DEGs induced by C48/80 in WT mice were mainly enriched in the following GO terms: response to stimulus, response to chemical,

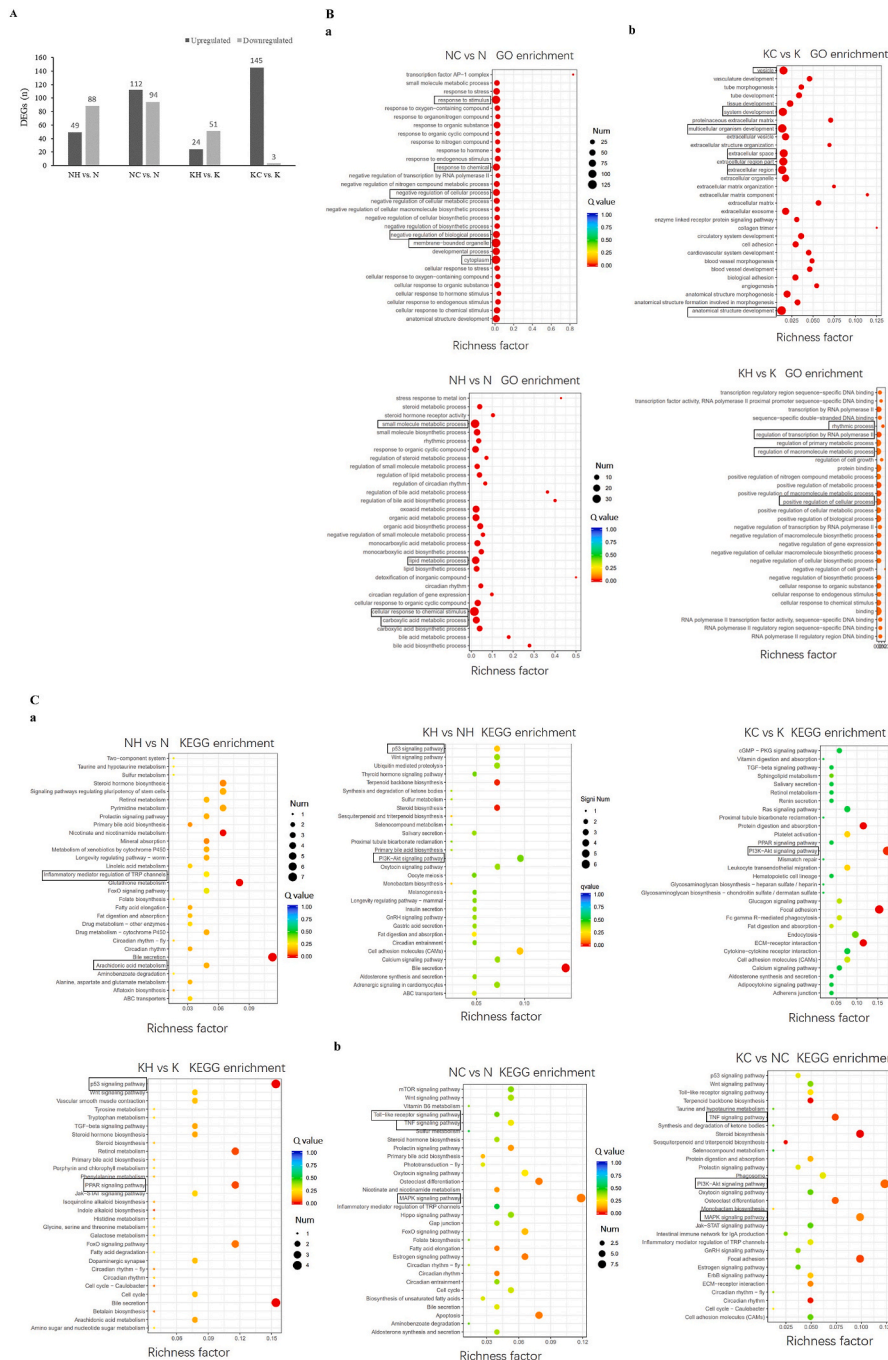


Fig. 4. Screening of genes with $q < 0.05$ and difference multiple >2 .

(A) Expression of various genes among different groups. (B) Scatter plots of significantly enriched functions of genes in wild-type (a) and knockout (b) mice. (C) Effects of *Mrgrb2* knockout on KEGG enrichment in mice administered with (a) *Houttuynia cordata* injection and (b) C48/80.

membrane-bounded organelle, negative regulation of cellular process, and negative regulation of biological process and cytoplasm. The HC injection-induced DEGs, on the other hand, were mainly enriched in small molecule metabolic process, lipid metabolic process, cell response to chemical stimulus, and carboxylic acid metabolic process (Fig. 4B).

In KO mice, C48/80-induced DEGs were enriched in functional classifications such as vesicle, system development, multicellular organism development, extracellular space, extracellular region, and anatomical structure development, whereas HC injection-induced DEGs were mainly enriched in the functional classifications of regulation of transcription by RNA polymerase II, rhythm process, positive regulation of cellular process, and regulation of macromolecular metabolic process

(Fig. 4B).

3.4.4. KEGG analysis of DEGs

We analyzed the KEGG pathway enrichment of DEGs, using $P < 0.05$, as the screening criterion (Table 2).

The DEGs in WT mice administered with HC injection were mainly enriched in nicotinate, nicotinamide metabolism, and glutathione metabolism pathways, whereas those in KO mice were mainly enriched in P53, bile secretion, retinol metabolism, peroxisome proliferators-activated receptors (PPARs), forkhead box-containing protein O sub-family (FOXO), and other signaling pathways. The DEGs in WT mice administered with C48/80 were mainly enriched in mitogen-activated

Table 2

Upregulated and downregulated KEGG pathways.

	Upregulated	Downregulated
NH vs N	Glutathione metabolism; Linoleic acid metabolism; Bile secretion; Drug metabolism-cytochrome P450; Arachidonic acid metabolism; Fat digestion and absorption; Inflammatory mediator regulation of TRP channels; ABC transporters; Steroid hormone biosynthesis; Retinol metabolism; Taurine and hypotaurine metabolism; Neurotrophin signaling pathway; Sulfur metabolism; Aldosterone synthesis and secretion	Nicotinate and nicotinamide metabolism; Mineral absorption; Primary bile acid biosynthesis; Signaling pathways regulating pluripotency of stem cells; Bile secretion; Prolactin signaling pathway; Aflatoxin biosynthesis; Alanine, aspartate and glutamate metabolism; Circadian rhythm-fly; Hippo signaling pathway; Folate biosynthesis; p53 signaling pathway
KH vs. K	Primary bile acid biosynthesis; PPAR signaling pathway; Porphyrin and chlorophyll metabolism; Fatty acid degradation; Retinol metabolism; Glycine, serine and threonine metabolism	Bile secretion; p53 signaling pathway; Indole alkaloid biosynthesis; FOXO signaling pathway; Cell cycle-Caulobacter; Beta lain biosynthesis; Circadian rhythm; Isoquinoline alkaloid biosynthesis; Dopaminergic synapse
KH vs NH	Steroid biosynthesis; Terpenoid backbone biosynthesis; Monobactam biosynthesis; Sesquiterpenoid and triterpenoid biosynthesis; p53 signaling pathway; Cell cycle; Synthesis and degradation of ketone bodies; Wnt signaling pathway; Selenocompound metabolism; Sulfur metabolism; Hippo signaling pathway; Primary bile acid biosynthesis; Jak-STAT signaling pathway; Focal adhesion; Butanoate metabolism	Bile secretion; Cell adhesion molecules (CAMs); Fat digestion and absorption; ABC transporters; Ubiquitin mediated proteolysis; Adrenergic signaling in cardiomyocytes; Salivary secretion; Gastric acid secretion; Calcium signaling pathway; Aldosterone synthesis and secretion
NC vs N	MAPK signaling pathway; Fatty acid elongation; Osteoclast differentiation; Toll-like receptor signaling pathway; FOXO signaling pathway; Apoptosis; TNF signaling pathway; Oxytocin signaling pathway	Nicotinate and nicotinamide metabolism; Signaling pathways regulating pluripotency of stem cells; Prolactin signaling pathway; Circadian rhythm; Estrogen signaling pathway; Wnt signaling pathway; Primary bile acid biosynthesis; Circadian rhythm-fly; Vitamin B6 metabolism; Folate biosynthesis; Aminobenzoate degradation; Hippo signaling pathway
KC vs K	ECM-receptor interaction; Leukocyte transendothelial migration; Sphingolipid signaling pathway; Fc gamma R-mediated phagocytosis; Focal adhesion; Platelet activation; Cell adhesion molecules (CAMs); PI3K-Akt signaling pathway; Fat digestion and absorption; Glucagon signaling pathway; Endocytosis; Focal adhesion	N-Glycan biosynthesis; Aldosterone synthesis and secretion
KC vs NC	Steroid biosynthesis; Terpenoid backbone biosynthesis; Sesquiterpenoid and triterpenoid biosynthesis; Focal adhesion; ECM-receptor interaction; PI3K-Akt signaling pathway; Protein digestion and absorption; Phagosome; Monobactam biosynthesis; Circadian rhythm; Cell cycle-Caulobacter; Cell adhesion molecules (CAMs); Intestinal immune network for IgA production; Circadian rhythm – fly	TNF signaling pathway; Osteoclast differentiation; MAPK signaling pathway; Inflammatory mediator regulation of TRP channels; GnRH signaling pathway; Circadian rhythm; Toll-like receptor signaling pathway; Insulin signaling pathway; Oxytocin signaling pathway; Aldosterone synthesis and secretion; Hematopoietic cell lineage; ErbB signaling pathway

protein kinase (MAPK), osteoclast differentiation, apoptosis, and estrogen signaling pathways; The DEGs in KO mice were mainly concentrated in signaling pathways, such as protein digestion and absorption, phosphatidylinositol 3-kinases/protein kinase B (PI3K/Akt), extracellular matrix (ECM) receptor interaction, and focal adhesion, among others (Fig. 4C).

The original transcriptome sequencing data have been uploaded to the SRA (Sequence Read Archive) database in NCBI (<https://www.ncbi.nlm.nih.gov/sra>). The data information is as follows: Submission ID: SUB10812773; BioProject ID: PRJNA791419.

3.5. Verification of transcriptome analysis

To facilitate the identification of mediators of *Mrgprb2* in drug induced anaphylactoid reactions, we focused on the genes whose expression exhibited significant alterations after treatment both in the mutant and wild type mice. Two genes involved in P53 and PI3K/Akt signaling pathways, *Sesn2* and *Reln*, were verified by real-time PCR. The results of qPCR showed that *Sestrin2* was downregulated by HC injection in WT mice, while *Mrgprb2* deletion could restore *Sestrin2* after HC injection. The expression of *Reelin* was diminished by C48/80, while *Mrgprb2* deletion could enhance *reelin* expression after C48/80 treatment. The data was presented in Supplement Fig 3.

4. Discussion

Adverse reactions caused by HC injection have attracted considerable attention recently. Elieh et al. (2020) found that HC injection induced toxins C3a and C5a by activating the complement system, and these toxins could combine with receptors on mast cells/basophils, resulting in anaphylactoid symptoms. Liu et al. (2010) found that HC injection might induce anaphylactoid reactions by stimulating the immune system and increasing the levels of the immunoglobulin IgG, IgA and IgM. However, the mechanism of the anaphylactoid reactions elicited by HC injection is still unclear because of the complex composition.

Recently, studies have reported that TCMIs such as baicalin (Wang et al., 2020) and sinomenine (Liu et al., 2017) can activate *Mrgprb2* and cause LAD2 cell degranulation. And our results further demonstrated that *Mrgprb2* was a key mediator in HC injection triggered mast cells degranulation, which lead to a drug dose-dependent β -hexosaminidase and histamine release.

In this study, the *Mrgprb2* KO mice were generated by CRISPR/Cas9 technology (Hu et al., 2021). Currently, our investigation on *Mrgprb2*-deficient mice would improve the understanding of human *MRGPRX2* (the orthologue of mouse *Mrgprb2*) functions in TCMIs induced anaphylactoid reactions. However, predicting functions of human *MRGPRX2* involved in anaphylactoid reactions based on mouse orthologue in animal experiments could lead to some misunderstandings due to species differences. Therefore, in the future research, the *MRGPRX2*-humanized model (mouse *Mrgprb2* replaced by human *MRGPRX2*) will be generated to investigate the role of *MRGPRX2* in TCMIs induced anaphylactoid reactions. This *Mrgprb2* knockout model will serve as the control strain.

To get close to the in vivo condition, the primary peritoneal mast cells were used to assess anaphylactoid reactions instead of Laboratory of allergic diseases 2 (LAD2) cells or human mast cell-1 (HMC-1) lines (Peter et al., 2016; Pundir et al., 2014). Our findings showed significantly decreased β -hexosaminidase and histamine levels in KO mice compared to those in WT mice injected with HC, which were generally consistent with those of previous reports that *Mrgprb2* knockout mice exhibited a reduced drug-induced inflammation effect. (Che et al., 2018).

To gain insight into roles of *Mrgprb2*, the transcriptional profiling and pathway screening were performed. The GO analysis showed that the four groups of DEGs were mainly concentrated in the functional classifications of stimulation response, small molecule, lipid, macromolecule, carboxylic acid metabolism, rhythmic process, and cell response to chemical stimulation, which were mainly basic stress and metabolic responses with no immune-related functions. The KEGG pathway analysis showed that the mechanisms of the anaphylactoid

reactions induced by HC injection and C48/80 are different. Based on the results, we focused on the genes involved in inflammation with significant alterations both in mutant and WT mice after treatment.

We focused on the *Sesn2* gene of P53 signaling pathway when studying the mechanism of anaphylactoid reaction caused by HC injection. *Sestrin2* (*Sesn2*), a highly conserved stress-inducible metabolic protein, can protect cells from genotoxicity and oxidative stress (Jun et al., 2013). Sestrins mainly inhibit the activity of mammalian rapamycin complex 1 target (mTORC1) by activating adenosine monophosphate dependent protein kinase (AMPK) (Eid et al., 2013). mTORC1 activator could suppress FcεRI-mediated mast cell degranulation and cytokine secretion (Rakhmanova et al., 2018). Besides, Amp-activated protein kinase (AMPK) is the main negative regulator of mTOR in cells. Studies indicate that *Sestrin2* inhibits mTOR activation in cells mainly through the activation of AMPK and phosphorylation of tuberous sclerosis 2 (TSC2) (Andrei and Michael, 2008). It has been reported that the activation of AMPK can inhibit mast cell FcεRI signal transduction and mast cell degranulation, thereby inhibiting allergic reaction (Hwang et al., 2013). We found that *Sestrin2* was downregulated and mTOR was following increased by HC injection in WT mice, while *Mrgprb2* deletion could restore *Sestrin2* after HC injection. Comparatively, the expression of AMPK was augmented when *Mrgprb2* was deleted in mice (Supplement Fig 4). From this standpoint, *Mrgprb2* could play a pivotal role in HC injection induced anaphylactoid reaction mediated by mTOR/AMPK pathway.

On the other side, in C48/80-induced anaphylactoid reaction, we found that *Reln* was upregulated possibly mediated by PI3K/AKT signaling pathway. *Reelin* (*Reln*) is a secreted extracellular matrix glycoprotein, which plays a more important role in the migration and localization of neurons in the developing brain (D'Arcangelo, G., 2005 and Niu et al., 2004). Castellano et al. (2015) found *Reelin* (*Reln*) as a key regulator of Ras-PI3-Kinase signaling downstream cell migration, also observed the induction of *Reelin*/E-cadherin axis. Although the role of *Reelin* in allergic reaction is still unknown, E-cadherin was found to mediate tissue interaction limited mast cell degranulation and inhibited allergic reactions in vivo and in vitro (Klewer et al., 2021). We found that, *Reelin* and E-cadherin were both upregulated when KO mice injected with C48/80 (Supplement Fig 4). Therefore, we speculated that *Mrgprb2* may be involved in the compound 48/80-induced allergic reaction mediated by the *Reelin*/E-cadherin axis. Based on our findings, the mechanisms of anaphylactoid reactions induced by C48/80 were different from HC injection. Therefore, we should be more careful to interpret data when we use C48/80 as a tool drug in the investigation of TCMI induced anaphylactoid reactions.

5. Conclusions

Mrgprb2 gene played a role in the anaphylactoid reaction induced by HC injection. In this study, RNA sequencing analysis provided new insights into the mechanism underlying the anaphylactoid reaction induced by HC injection and C48/80.

CRediT authorship contribution statement

Xinyu Sun: Investigation, Data curation, Writing – original draft. **Wanlin Lv:** Investigation, Data curation, Writing – original draft. **Yalan Wang:** Investigation. **Xiao Zhang:** Investigation. **Zhen Ouyang:** Conceptualization, Methodology, Writing – review & editing. **Runting Yin:** Conceptualization, Methodology, Writing – review & editing. **Yuan Wei:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2022.115053>.

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