

The Mutation of Ednrb(c.857 T > C) Associated With Atrophied Spleen Phenotype in Mice

Fei Wu

College of Animal Science and Technology, Northwest A&F University

Mei Li

College of Animal Science and Technology, Northwest A&F University

Fu-Wen Wang

College of Animal Science and Technology, Northwest A&F University

Yuan Gao

College of Animal Science and Technology, Northwest A&F University

Tariq Munir

Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture Faisalabad

Zi-hui Zhou

College of Animal Science and Technology, Northwest A&F University

Chuzhao Lei

College of Animal Science and Technology, Northwest A&F University

Rui-Hua Dang (✉ dangruihua@nwsuaf.edu.cn)

college of animal science and technology, northwest A&F university

Research article

Keywords: spleen, EDNRB, sequencing, KEGG, Hippo signaling pathway

Posted Date: February 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-229607/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: The endothelin signaling pathway plays an important function in the migration, proliferation, and differentiation of neural crest cells. Endothelin receptor B (*EDNRB*) was reported to have a small spleen phenotype in its deficient mouse model. In our study, we also found that the mutation of *EDNRB* gene (c. 857 T > c) led to an atrophied spleen phenotype in mice. Different genotypes of *EDNRB* were significantly correlated with the spleen-kidney ratio, and the spleen phenotypes of *Ednrb*^{m1yzcm} mice were smaller. The results of the tissue section and H&E staining showed that the spleen microstructure of *Ednrb*^{m1yzcm} mice was abnormal. In order to explore the molecular mechanism, three groups of *Ednrb*^{m1yzcm} and wild-type mice were used as control, and standard $|\log_2(\text{FoldChange})| > 1$ and $Padj < 0.05$ were used to study the influence of *EDNRB* gene mutation on spleen transcriptional group in mice. GO and KEGG enrichment analysis was conducted to explore the signal pathway related to small spleen phenotype.

Results: Through sequencing of mouse spleen transcriptome, 121 differentially expressed genes were selected. Results of the KEGG pathway enrichment analysis showed that in *Ednrb*^{m1yzcm} mice, upregulated genes were significantly enriched in the Hippo signaling pathway, this pathway inhibits cell growth and modulates organs size and volume; and down-regulation of immune functionally associated pathways such as cytokine receptor interaction and chemokine signaling pathway. In addition, chemokine of Chemokine signaling pathway may also be related to the development of spleen immune tissue structure.

Conclusions: In the experiment, we found that mice with mutations in the *EDNRB* gene have features such as an atrophied spleen and changes in the structure of the spleen. In order to explore the reasons, we performed RNA sequencing on three groups of *Ednrb*^{m1yzcm} and wild-type mice, and we found that upregulated genes were significantly enriched in the Hippo signaling pathway. And this signal Pathway is a proven signaling pathway that controls organ size and immune function, so we speculate that the size of the spleen may be related to the Hippo signaling pathway. This study provides a theoretical study of the mechanism of spleen development.

Background

The growth of the spleen begins in the fifth week of development, and it originates from the mesoderm of the embryonic viscera until lymphatic colonization. The spleen acts as the hematopoietic organ[1] turning out to be the largest peripheral lymphatic organ in the body. When its organizational development completes, its structure and function becomes very complex. The essence of the spleen is distributed into three portions: white pulp, red pulp and marginal zone[2–4]. The white pulp is composed of dense lymphocytes, which is the primary site for the body's specific immunity[5]. When the antigen invades the spleen and mediates the humoral immune response, a number of lymph nodes in the white pulp increases. The red pulp of the spleen is mainly composed of blood sinus and splenic cord. The slow rate of blood flow in the red pulp facilitates the complete antigens and phagocytes interaction. Marginal zone

(MZ) is located at the junction of red and white medulla. The lymphocytes in this area are sparse compared with white medulla, mainly B cells, but there are more macrophages. It is an essential site for antigen capture, antigen recognition and immune response induction in the spleen[6]. Immune function is the most crucial function of the spleen. The interactions between various immune cells and immune factors in the spleen restrict each other. It can complete the body's non-specific immune functions through phagocytosis, and T cells and B cells mediated the cellular and humoral immunity to exert specific immune functions.

The mouse *EDNRB* gene is located on the negative strand of chromosome 14 and contains 29025bp[7, 8]. It plays an important role in the pathogenesis of congenital megacolon, melanoma and other related diseases. This gene encodes a G protein-coupled receptor and is localized to the cell membrane under physiological conditions. As a G protein-coupled receptor superfamily [9], it binds to endothelin (ET) and transmits information into the cell through the second messenger system of intracellular Ca²⁺ + phospholipid-dependent protein kinase to promote endoplasmic reticulum and sarcoplasm[10, 11]. There is a quick surge of Ca²⁺ in the reticulum calcium reservoir, and Ca²⁺ concentration in the cytoplasm rapidly increases [12]. During embryonic development, the *EDNRB* / *EDN3* / *ECE1* signaling pathway plays an important role in the migration and development of neural crest cells into ganglion cells, affecting the formation of the colorectal and enteric nervous system[13–16]. Previous experiments have also shown that the gene affects the size of the spleen[17]. This experiment will explore the changes in the spleen phenotype after mutation of the *EDNRB* gene (c.857 T > C). In this experiment, a male mouse with abdominal leukoplakia whose T - C mutation at base 857 of the coding region of the *EDNRB* gene was obtained by ENU mutagenesis screening, and the offspring were obtained by cross-breeding with ordinary female mice, and their tail DNA was collected. As a template, homozygous mice (*Ednrb*^{m1yzcm} mice) were genotyped by PCR and sequencing technology. Transcriptome sequencing was performed on homozygous mice and wild-type mice to explore their differentially expressed genes and enrichment pathways, to obtain theoretical evidence of the effect of this gene mutation on spleen size in mice.

Results

Phenotype of homozygous mutant(*Ednrb*^{m1yzcm} mice) and its small spleen phenotype

A heterozygote male mouse with abdominal white spot was mutated in *EDNRB* gene (c.857 T > C) which obtained by ENU mutagenesis screening. The homozygous mice (*Ednrb*^{m1yzcm} mice) were obtained by genotyping and cross breeding between heterozygous mice with mutations, as shown in figure (Figure 1A). *Ednrb*^{m1yzcm} mice have irregular black coats on the head and tail, and the hair color in the other parts was white.

After dissecting different genotypes mice at the age of 9 days, we found significant differences in spleen size (Figure 1B). Spleen tissue sections and H&E staining (Harris hematoxylin and eosin-y staining) were performed on mice having different genotypes(Figure 1C). Result demonstrates that the spleen microarchitecture structure of *EDNRB*^{m1yzcm} mice was abnormal, compared with wild-type mice, the white

and red pulps of the spleen were significantly decreasing. The white pulp was stained by blue, and the red pulp is stained by red. In the spleen of normal mice, the white pulp follicular mass and the surrounding scattered red pulp were visible, while the spleen of homozygous mice (*Ednrb*^{m1yzcm} mice) lacked the white pulp follicular structure, and the loss of red pulp is more pronounced.

The genotype of *Ednrb*^{m1yzcm} mutant was significantly correlated with the spleen-kidney ratio

Spleen and kidney of 5 groups of mice with different genotypes were randomly selected for weighing and statistical analysis. As shown in Table 1, the spleen and kidney weights of *Ednrb*^{m1yzcm} mice (CC type) are significantly smaller than mutant heterozygous (CT type) and wild type (TT type). Therefore, *EDNRB* gene mutation (c.857 T > C) is related to the size of spleen and kidney in mice. The spleen-kidney ratios of *Ednrb*^{m1yzcm} mice (CC) were significantly lower than those of mutant heterozygous (CT type) and wild type (TT type). SPSS analysis revealed a P-value of 0.005 that denotes the significant difference. Thus, *EDNRB* gene mutation (c.857 T > C) is related to the development of spleen size in mice.

Differential gene expression in *EDNRB*^{m1yzcm} mice and wild-type mice

The differential genes of all the comparison groups were merged and used as a differential gene set, the different gene sets were clustered, and the genes with similar expression patterns were clustered [Additional file 1]. The mainstream hierarchical clustering was used for clustering analysis of genes' FPKM values, as shown in the clustering heat map(Figure 2A). HM (*EDNRB*^{m1yzcm} mice) and WT (wild-type mice) groups could be seen as two different treatment groups with significant differences in gene expression.

According to the overlap of differentially expressed genes between different comparative combinations, Venn diagram was drawn to screen the differentially expressed genes common or unique to certain comparative combinations. The comparative combination treatment group and control group were expressed genes Wayne figure, as shown in Figure 2B. The *Ednrb*^{m1yzcm} mutant and wild-type mice had a total of 12,359 differentially expressed genes [Additional file 2]. However, by adopting the use of standard $|\log_2(\text{FoldChange})| > 1$ & $P_{adj} < 0.05$ after screening, 121 differentially expressed genes, comprising of 85 upregulated genes and 36 down-regulated genes was revealed, as shown in Figure 2C.

Genes differentially expressed in mice with different genotypes are enriched in the Hippo signaling pathway that are related to the small spleen phenotype of mice

From the results of GO (Gene Ontology; <http://geneontology.org/>) enrichment analysis [Additional file 3 sheet 1], the most significant 30 terms were selected to draw histogram (Figure 3A). Biological processes was primarily focused on the cellular response to extracellular stimulus and amine metabolism collagen metabolic process, multicellular organic macromolecule metabolic process and other functions related to cellular response and metabolism. Cell composition was principally concentrated in pre-autophagosomal structure, lytic vacuole and lysosome, which may be due to the immune function of spleen tissues. Molecular functions are specifically concentrated on heat shock protein binding, Oxidoreductase activity,

cysteine-type endopeptidase inhibitor activity involved in apoptotic process etc. It is mainly associated with enzymes redox activity, binding of various molecules and apoptosis.

KEGG(Kyoto Encyclopedia of Genes and Genomes; <https://www.genome.jp/kegg/>) is a comprehensive database integrating genomic, chemical and systematic functional information. With *Padj* less than 0.05 as the threshold of significant enrichment, the most significant 20 upregulated KEGG pathways were selected from the KEGG enrichment results[Additional file 3 sheet 2] to draw a histogram for display (Figure 3B). Upregulated genes were significantly mediated in p53 signaling pathway, Hippo signaling pathway, beta-alanine metabolism, characterization-multiple species, Arginine and proline metabolism, Amino sugar and nucleotide sugar metabolism, where Hippo signaling pathway inhibits cell growth, and upstream membrane protein receptors in the Hippo signaling pathway act as receptors for extracellular growth inhibition signals. Pan D suggested the core Hippo kinase cascade integrates multiple upstream feedbacks, that enabling dynamic regulation of tissue homeostasis in animal development and physiology[18]. Once the extracellular growth inhibition signals are sensed, a cascade of kinase phosphorylation reactions are activated and terminating the phosphorylation of downstream effectors YAP and TAZ. Cytoskeletal proteins binds to phosphorylated YAP and TAZ, holding it in the cytoplasm and reducing its nuclear activity, thus attaining the regulation of organ size and volume. Therefore, it could be associated to the reduction of spleen tissue.

The 20 most significantly down-regulated gene enrichment pathways were selected(Additional file 3 sheet 3, Figure 2A). Predominantly selected pathways are Cytokine-Cytokine receptor interaction, Osteoclast differentiation and Histidine metabolism, Renin - angiotensin system, RNA polymeras, Fatty acid elongation, Chemokine signaling pathway. Cytokine-Cytokine receptor interaction and Chemokine signaling pathway are of significant importance in guiding immune cells to and within lymphoid and non-lymphoid tissues[19]. Chemokine of Chemokine signaling pathway is a family of small molecular cytokines whose primary function is to attract immune cells to the inflammatory site[20]. This signaling pathway highlights different types of Chemokine and its receptors, as well as how Chemokine signaling activates the JAK/STAT, Ras, ERK and Akt pathways. These down-regulated genes in pathways associated with immune function may be related to the development of immune tissue structures in the spleen..

Discussion

The mice used in this study were mutant mice with *EDNRB* gene, and *EDNRB* was one of the primary susceptible genes for congenital megacolon. Moreover, subsequent anatomical experiments all confirmed that homozygous mutant mice with *Ednrb*^{m¹yzcm} had congenital megacolon in addition to changes in hair color and reduced spleen. Zhi Cheng reported that mice with genetic deficiency of the endothelin receptor type B displays not just colonic aganglionosis, but also manifest a profoundly abnormal immunophenotype characterized by markedly small spleens, B and T cell splenic lymphopenia, and disordered splenic microarchitecture[21]. This finding is consistent with our experimental results, which shows that the loss of function of *EDNRB* gene can affect the development of spleen as well as the

congenital megacolon. Dang et al. reported that the AGH-Ednrb^{sl/sl} rat model of HSCR displayed lymphoid depletion with significantly reduced spleen weight and total splenic cell count of less than 15% in wild-type AGH rats, with severe B, CD4 and CD8 T cells reduction [17]. In conclusion, we have shown that the *Ednrb*-null mutation not only presents as colonic inactivation similar to Hirschsprung's disease, but also presents as an immune abnormality characterized by significant reduction in spleen volume.

Similar to the T cell homing to the tumor's endothelial barrier[22], Frykman noted that the spleen was smaller in *Ednrb*^{-/-} bone marrow transplant recipients, suggesting that *EDNRB* may play a role in lymphocyte transport between bone marrow, thymus, and spleen[23]. However, the lymphocytic recirculation pathway through the spleen remains a mystery, and the lymphocytic migration pathway to the spleen is still undefined[2]. Gosain A found that *Ednrb*^{NCC^{-/-}} showed reduced spleen size and structural changes, decreased red medulla in myeloid center and marginal areas, and decreased B lymphocytes, especially IgM⁺ IgD^{hi} (mature) B lymphocytes, which are usually activated after antigen presentation and produce IgA[24]. The spleen is the primary source of IgA producing mature B lymphocytes; therefore the spleen may be the primary anatomic site of B-lymphocyte defects in *Ednrb*^{NCC^{-/-}}.

KEGG analysis results showed that the down-regulated genes were significantly enriched in the Hippo signaling pathway. Originally discovered in *Drosophila*, the Hippo signaling pathway is considered to be a conserved signaling pathway that controls organ size during development by inhibiting cell growth and proliferation and promoting apoptosis. The Hippo pathway acts in repressing the transcription of target genes promoting proliferation and survival. And more recently, there is growing evidence that Hippo signals play a crucial role in regulating the immune system. To date, various knockout mice of major components of the Hippo pathway have been elucidated the role of this pathway in the immune system. Hippo signaling pathway has been extensively studied in lymphocytes, especially T cells, and emerging results show that MST1/2 (MST1/2 kinases, the mammalian orthologs of *Drosophila* Hippo.) is critical for T-lymphocyte development, migration, homing, and differentiation. MST1 also regulates adhesion and trafficking of B-cell; the number of peripheral B cells, as well as splenic marginal zone B cells, was significantly reduced in MST1-deficient mice[25, 26]. All of these suggest that the major reason for the shrinkage of the spleen is probably the effect of this signaling pathway.

Conclusions

In this study, RNA sequencing was performed on three groups of *Ednrb*^{m1yzcm} and wild-type mice. The methods of KEGG pathway enrichment analysis and GO enrichment analysis enriched differentially expressed genes in different signaling pathways. We found that upregulated genes were significantly enriched in Hippo signaling. This signaling pathway is a proven signaling pathway that controls organ size and immune function. Therefore, we speculate that the size of the spleen may be related to the Hippo signaling pathway. Down-regulated genes are mainly enriched in Cytokine-Cytokine receptor interaction, Chemokine signaling pathway, etc. These signal pathways related to the immune function of the spleen, and the spleen is mainly composed of lymphocytes and other cells, so while the immune function is

affected, it also has a certain impact on the size of the spleen. This study provides a theoretical study for the development of the spleen.

Methods

Animal

Male adult mice (SPF grade, 7-9 weeks old) with *EDNRB* mutation induced by ENU were purchased from College of Veterinary medicine Yangzhou University. All the mice were caged in a controlled environment. All procedures were approved by the Committee for the Ethics on Animal Care and Experiments in Northwest A&F University. To minimize suffering during sacrifice, mice were euthanized by cervical dislocation. A total of 269 mice were bred until the follow-up test, of which 23 were used in the specific experiment: Five individuals of CC type (homozygous mutant *Ednrb*^{m1yzcm} mice), CT type and TT type (wild type mice, control group) were randomly selected to analyze the correlation between genotype and spleen-kidney ratio; Two individuals of CC type and TT type (control group) were randomly selected for tissue sections and HE staining; Three individuals of CC type and TT type (control group) were randomly selected for RNA sequencing. The remaining 246 were euthanized because the genotype did not meet the breeding and testing requirements.

Acquisition of *Ednrb*^{m1yzcm} mice

Three male mouse with abdominal white spot mutation were obtained by ENU mutagenesis screening , we mated them with normal female mice (N=20). female mice (wild type) in estrus are placed into the cages with male adult mice with *EDNRB* mutation (two females in each cage with one male), the next morning, check the female for vaginal plugs to make sure the mating is successful. phenotyping data from both sexes is collected at regular intervals on age-matched wild-type mice of equivalent genetic backgrounds (This part of the work was done by F.W). The animals were randomly assigned to mate after being genotyped, using a computer-based random sequence generator. The heterozygous male (CT N=21) and female (CT N=32) offspring mice with *EDNRB* gene mutation were cultured to estrus and mated to obtain the second generation of offspring mice. The mouse tail tissue was collected. The mice tail tissue DNA was extracted using the improved phenol–chloroform method [27], PCR technology was used to amplify the gene fragment containing the mutation site (the 857th base T to C of the *EDNRB* gene coding region), and genotyping was performed by sequencing technology to select heterozygotes containing mutations. The heterozygous male and female offspring mice with *EDNRB* gene mutation were cultured to estrus and mated to obtain the second generation of offspring mice. To minimize suffering during sacrifice, mice without mutations were euthanized by cervical dislocation. The mice fasted for 18 hours before autopsy, but were provided with normal drinking water, The mice were euthanized at the operating table. The mutant heterozygous mice were selected for hybridization, and the *Ednrb*^{m1yzcm} mice with irregular black spots on head and hip and mice with white body were obtained. The size and sequence of the primers, and T_m (°C) are shown in Table 2.

Anatomy and organ weighing of Ednrb^{m1yzcm} mice and wild type mice

We weighed Ednrb^{m1yzcm} mice and wild-type mice that were genotyped by sequencing at 9th day of birth, with an electronic balance, dissected their spleen and kidneys, weighed and recorded the data (This part of the work was done by M.L). Three spleens of Ednrb^{m1yzcm} mice and three spleens of wild-type mice were rapidly stored in the refrigerator at -80°C (for subsequent transcriptome sequencing). One spleen from both Ednrb^{m1yzcm} mice and wild-type mice were placed in the tissue fixation solution (for the preparation of subsequent tissue sections).

Correlation analysis of genotype of Ednrb^{m1yzcm} mutant mice and spleen-kidney ratio

After 9 days of birth, the second-generation mice tail tissue was collected and their DNA was extracted as a template, and the gene fragment containing the mutation site (base 857 T to C in *EDNRB* gene coding region) was amplified by PCR. And then we genotyped by sequencing technology. Five individuals of CC type (homozygous mutant Ednrb^{m1yzcm} mice), CT type and TT type were randomly selected. SPSS 19.0 software (Statistical Product and Service Solutions, Version 19.0 Edition, IBM, Armonk, NY, USA) for one-way analysis of variance (ANOVA) was used to analyze the significant relationship between different genotypes and ratio of their spleen weight to kidney weight and to know whether the base T to C mutation is related to the spleen-kidney ratio of mice (This part of the work was done by F.W.W).

Production of tissue sections.

DNA was collected from the second-generation mice tail tissue on 9th day after birth, and the gene fragment containing the mutation site (base 857 T to C in *EDNRB* gene coding region) was amplified by PCR technology. An individual of CC type (Ednrb^{m1yzcm} homozygotic mutant) and an individual of TT type were selected, their spleen tissues were dissected and collected, soaked in tissue fixation solution, paraffin sections were prepared, H&E (Harris hematoxylin and eosin-y) staining was conducted, and tissue section graphs were photographed with 20× and 40× resolution using CaseViewer software.

Construction and sequencing of spleen tissue

The spleens of 3 Ednrb^{m1yzcm} mutant homozygous mice (HM1, HM2, HM3) and 3 normal wild-type mice (WT1, WT2, WT3) were sent to Novogene Co., Ltd. Standard extraction method was used to extract RNA from spleen tissues. NanoPhotometer spectrophotometer was used to detect RNA purity (OD260/280 and OD260/230 ratios) and Agilent 2100 bioanalyzer was used to accurately detect RNA integrity. After taking strict quality control measures according to the two quality control standards, library construction, quality inspection and computer sequencing were conducted.

Collation and analysis of sequencing data of spleen tissue

Differential gene expression analysis

Differential gene analysis was performed according to the screening method of differentially expressed genes of Novogene Co., Ltd. The standard was $|\log_2(\text{FoldChange})| > 1$ & $P_{adj} < 0.05$, and the differentially expressed multiple was located at 2 times or more, with p_{adj} value set below 0.05.

GO function and KEGG pathway enrichment analysis

ClusterProfiler software was used to analyze the GO function enrichment and KEGG pathway enrichment of differential gene sets. The target gene set (differential gene set) is mapped to the background gene set. The enrichment analysis is based on the principle of hypergeometric distribution, in which the differential gene set is a differential gene obtained by significant difference analysis and marked to the GO and KEGG database gene set. Background gene sets are all sets of genes that were analyzed for significant differences and marked to GO or KEGG databases. $P_{adj} < 0.05$ was used as the threshold for significant enrichment.

Abbreviations

EDNRB: Endothelin receptor B; H&E staining: hematoxylin-eosin staining; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; ENU: ethyl nitrosourea; SPSS: Statistical Product and Service Solutions; FPKM: Reads Per Kilobase of exon model per Million mapped reads; YAP and TAZ: Yes-associated protein (YAP)/transcriptional coactivator with a PDZ-binding domain (TAZ); JAK/STAT: Janus Kinase-Signal Transducer and Activator of Transcription; Ras: rat sarcoma virus; ERK: signal-regulated kinase; Akt: protein kinase B; p53 signaling pathway: the tumor suppressor gene tumor protein 53; MST1/2: MST1/2 kinases, the mammalian orthologs of Drosophila Hippo; PCR: polymerase chain reaction; TM: temperature; NCC: neural crest cell; EdnrB^{NCC}^{-/-}: EdnrB was deleted from the neural crest; IgA: immunoglobulin A

Declarations

Ethics approval and consent to participate

All procedures involving animals conformed to Guidelines on Humane Treatment of Laboratory Animals by the Ministry of Science and Technology of China and were approved by the Committee for the Ethics on Animal Care and Experiments in Northwest A&F University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and Additional files.

Competing interests

The authors declare no conflict of interest.

Funding

This research was funded by National Natural Science Foundation of China , grant number 81770514. The funding bodies had not role in the design of the study and collection, analysis, and interpretation of data or in writing the manuscript.

Authors' contributions

Conceptualization, F.W. and M.L.; methodology, F.W.; software, F.W.W.; validation, Y.G. and Z.Z.; formal analysis, M.L.; investigation, F.W.W.; resources, R.D. and C.Z.L.; data curation, F.W.; writing—original draft preparation, F.W.; writing—review and editing, T.M. and R.D.; visualization, Y.G.; supervision, R.D.; project administration, F.W.; funding acquisition, R.D. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

We thank Dr. Chen Bing of Yangzhou University to donate Ednrb^{m1yzcm} mice.

References

1. Brendolan A, Rosado MM, Carsetti R, Selleri L, Dear TN. Development and function of the mammalian spleen. *BioEssays: news reviews in molecular cellular developmental biology*. 2007;29:166–77. doi:10.1002/bies.20528.
2. Steiniger BS. Human spleen microanatomy: why mice do not suffice. *Immunology*. 2015;145:334–46. doi:10.1111/imm.12469.
3. Kashimura M, Shibata A. [Structure and functions of the human spleen: relationship between microcirculation and splenic functions]. [*Rinsho ketsueki*] *The Japanese journal of clinical hematology*. 1989;30:1234–8.
4. Cesta MF. Normal structure, function, and histology of the spleen. *Toxicol Pathol*. 2006;34:455–65. doi:10.1080/01926230600867743.
5. Steiniger B, Ruttinger L, Barth PJ. The three-dimensional structure of human splenic white pulp compartments. *The journal of histochemistry cytochemistry: official journal of the Histochemistry Society*. 2003;51:655–64. doi:10.1177/002215540305100511.
6. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005;5:606–16. doi:10.1038/nri1669.
7. Amiel J, Attie T, Jan D, Pelet A, Edery P, Bidaud C, Lacombe D, Tam P, Simeoni J, Flori E, et al. Heterozygous endothelin receptor B (EDNRB) mutations in isolated Hirschsprung disease. *Human molecular genetics*. 1996;5:355–7. doi:10.1093/hmg/5.3.355.

8. Puffenberger EG, Hosoda K, Washington SS, Nakao K, deWit D, Yanagisawa M, Chakravart A. A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell*. 1994;79:1257–66. doi:10.1016/0092-8674(94)90016-7.
9. Oue T, Puri P. Altered endothelin-3 and endothelin-B receptor mRNA expression in Hirschsprung's disease. *Journal of pediatric surgery*. 1999;34:1257–60. doi:10.1016/s0022-3468(99)90163-x.
10. Chen YS, Liu F, Luo YH, Fan Y, Xu FG, Li P, Zhou B, Pan XY, Wang CC, Cui L. EDNRB isoform 3 confers Temozolomide resistance in A375 melanoma cells by modulating membrane potential, reactive oxygen species and mitochondrial Ca(2). *Cancer management research*. 2019;11:7353–67. doi:10.2147/cmar.s208604.
11. Houde M, Desbiens L, D'Orleans-Juste P. Endothelin-1: Biosynthesis, Signaling and Vasoreactivity. *Adv Pharmacol*. 2016;77:143–75. doi:10.1016/bs.apha.2016.05.002.
12. Kang HY, Kang WH, Lee C. Endothelin-B receptor-mediated Ca²⁺ signaling in human melanocytes. *Pflug Arch: Eur J Physiol*. 1998;435:350–6. doi:10.1007/pl00008082.
13. Barlow A, de Graaff E, Pachnis V. Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. *Neuron*. 2003;40:905–16. doi:10.1016/s0896-6273(03)00730-x.
14. Woodward MN, Kenny SE, Vaillant C, Lloyd DA, Edgar DH. Time-dependent effects of endothelin-3 on enteric nervous system development in an organ culture model of Hirschsprung's disease. *Journal of pediatric surgery*. 2000;35:25–9. doi:10.1016/s0022-3468(00)80007-x.
15. Bondurand N, Dufour S, Pingault V. News from the endothelin-3/EDNRB signaling pathway: Role during enteric nervous system development and involvement in neural crest-associated disorders. *Dev Biol*. 2018;444(Suppl 1):156-s169. doi:10.1016/j.ydbio.2018.08.014.
16. Butler Tjaden NE, Trainor PA. The developmental etiology and pathogenesis of Hirschsprung disease.
17. Dang R, Sasaki N, Nishino T, Nakanishi M, Torigoe D, Agui T. Lymphopenia in Ednrb-deficient rat was strongly modified by genetic background. *Biomed Res (Tokyo Japan)*. 2012;33:249–53. doi:10.2220/biomedres.33.249.
18. Pan D. The hippo signaling pathway in development and cancer. *Developmental cell*. 2010;19:491–505. doi:10.1016/j.devcel.2010.09.011.
19. Zhang X, Liu F, Bai P, Dong N, Chu C. Identification of key genes and pathways contributing to artery tertiary lymphoid organ development in advanced mouse atherosclerosis. *Mol Med Rep*. 2019;19:3071–86. doi:10.3892/mmr.2019.9961.
20. Schulz O, Hammerschmidt SI, Moschovakis GL, Forster R. Chemokines and Chemokine Receptors in Lymphoid Tissue Dynamics. *Annu Rev Immunol*. 2016;34:203–42. doi:10.1146/annurev-immunol-041015-055649.
21. Cheng Z, Wang X, Dhall D, Zhao L, Bresee C, Doherty TM, Frykman PK. Splenic lymphopenia in the endothelin receptor B-null mouse: implications for Hirschsprung associated enterocolitis. *Pediatr Surg Int*. 2011;27:145–50. doi:10.1007/s00383-010-2787-y.

22. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, Katsaros D, O'Brien-Jenkins A, Gimotty PA, Coukos G. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. *Nature medicine*. 2008;14:28–36. doi:10.1038/nm1699.
23. Frykman PK, Cheng Z, Wang X, Dhall D. Enterocolitis causes profound lymphoid depletion in endothelin receptor B- and endothelin 3-null mouse models of Hirschsprung-associated enterocolitis. *Eur J Immunol*. 2015;45:807–17. doi:10.1002/eji.201444737.
24. Gosain A, Barlow-Anacker AJ, Erickson CS, Pierre JF, Heneghan AF, Epstein ML, Kudsk KA. Impaired Cellular Immunity in the Murine Neural Crest Conditional Deletion of Endothelin Receptor-B Model of Hirschsprung's Disease. *PLoS One*. 2015;10:e0128822. doi:10.1371/journal.pone.0128822.
25. Hong L, Li X, Zhou D, Geng J, Chen L. Role of Hippo signaling in regulating immunity. *Cell Mol Immunol*. 2018;15:1003–9. doi:10.1038/s41423-018-0007-1.
26. Katagiri K, Ohnishi N, Kabashima K, Iyoda T, Takeda N, Shinkai Y, Inaba K, Kinashi T. Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. *Nature immunology*. 2004;5:1045–51. doi:10.1038/ni1111.
27. Clements DN, Wood S, Carter SD, Ollier WER. Assessment of the quality and quantity of genomic DNA recovered from canine blood samples by three different extraction methods. *Res Vet Sci*. 2008;85:74–9. doi:10.1016/j.rvsc.2007.09.009.

Tables

Table 1. Correlation between genotype of *Ednrb*^{m1yzcm} mutant mice and spleen-kidney ratio.

Project	Genotype			P value
	CC	CT	TT	
Spleen weight	0.015±0.006a	0.032±0.006b	0.028±0.003a	0.015
Kidney weight	0.042±0.012a	0.064±0.013b	0.061±0.008a	0.001*
Spleen-kidney ratio	0.34±0.07a	0.49±0.06b	0.46±0.06a	0.005*

Note : LSM ± SE, least-squares means and their standard errors for each genotypic class reported. a,b Means significantly different for genotype frequencies with genetic groups (*, p < 0.05)

Table 2. Information on primer sets used in this study. F—forward; R—reverse.

Primer Sequences (5'–3')	Primer Size (bp)	Tm (°C)
F: GGCATTTGTAATTGACATTTGATAC	25	57
R: ATACTCTACTAGCTTCTTCCATTCT	25	

Figures

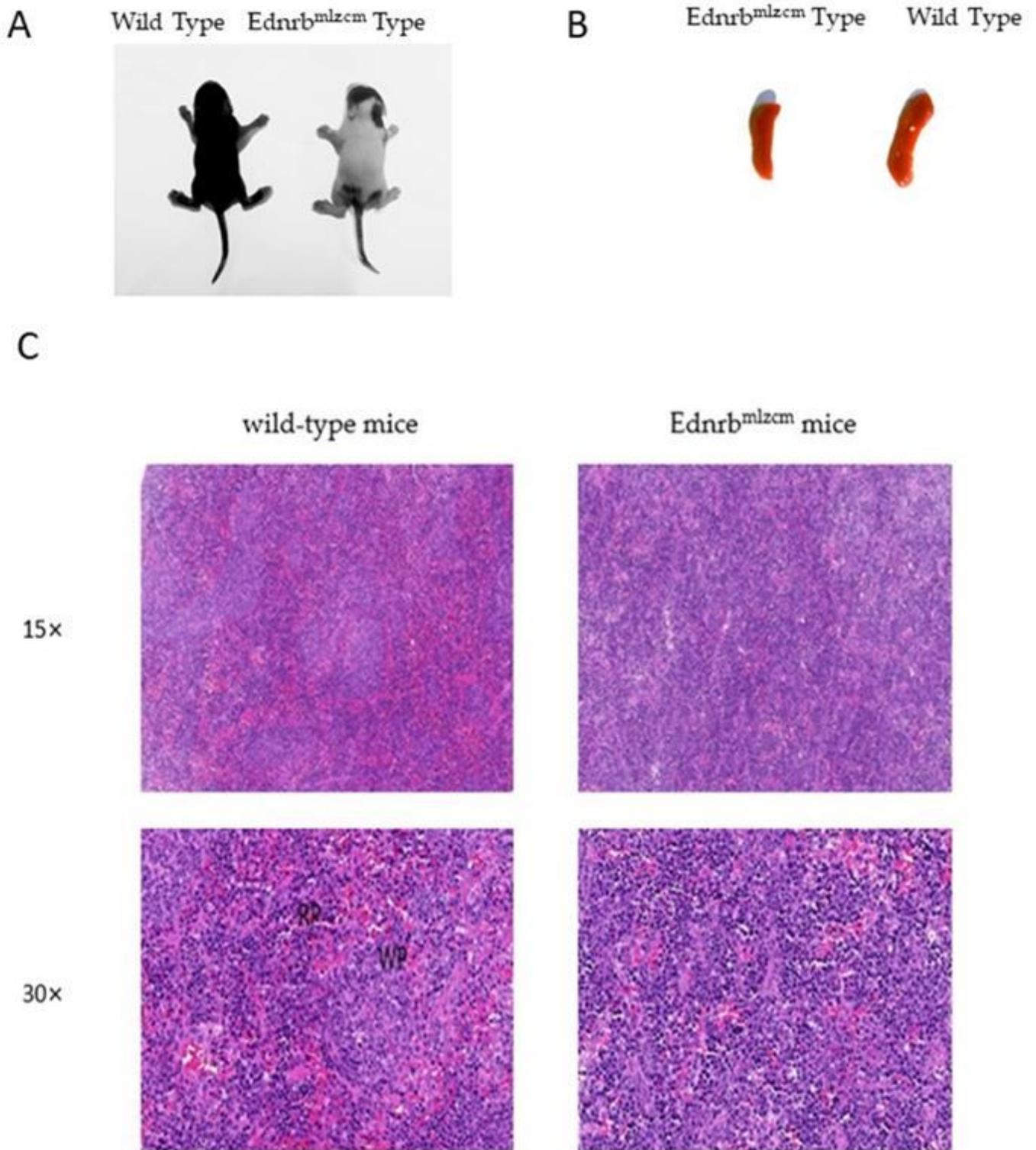


Figure 1

EDNRB gene mutation resulted in small spleen phenotype in mice. (a) EDNRBm1yzcm mice and wild type mice at the postnatal 9 day. (b) Representative picture showing the small spleen of the EDNRBm1yzcm mice comparing to the wild type mice. (c) Hematoxylin and eosin staining with a significant reduction of the white pulp(WP), red pulp(RP), trabecular in the EDNRBm1yzcm mice compared to wild type mice at the postnatal 9 day.

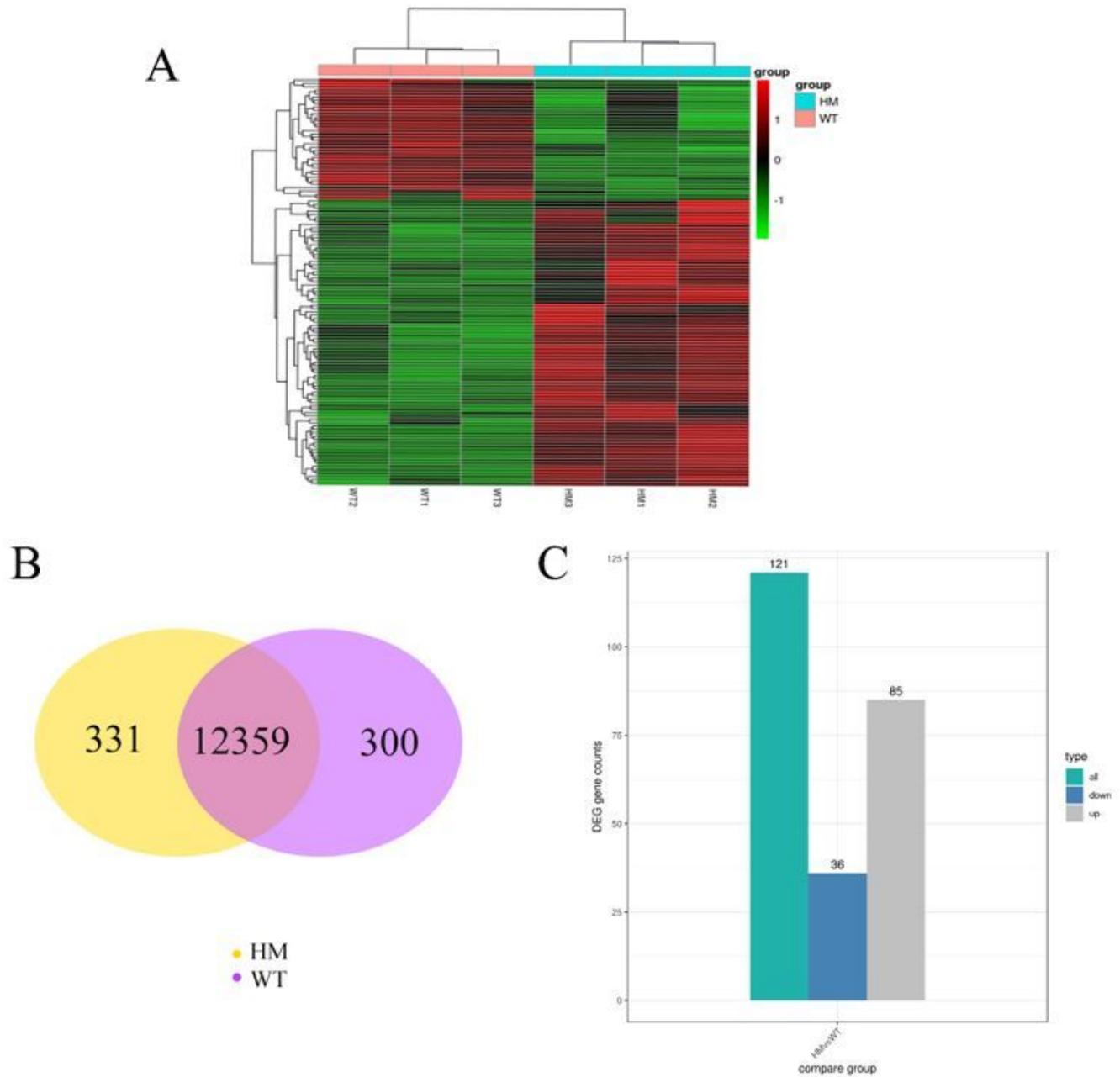


Figure 2

Different genotypes of mice showed differential gene expression. (a) Heat map of a cluster of differentially expressed genes. The abscissa in the figure is the sample name, and the ordinate is the normalized value of FPKM. The deeper the red color, the higher the expression and the deeper the green color, the lower the expression. (b) Differential gene Venn diagram. The sum of all the numbers in the

circle in the Venn diagram represents the total number of differentially expressed genes in the comparison combination, and the overlapping region represents the number of differentially expressed genes in common between the combinations. (c) Statistical bar chart of the number of differentially expressed genes in different combinations. Grey and blue represents upregulated, and down-regulated differentially expressed genes respectively, and the Numbers on the column represent the number of differentially expressed genes.

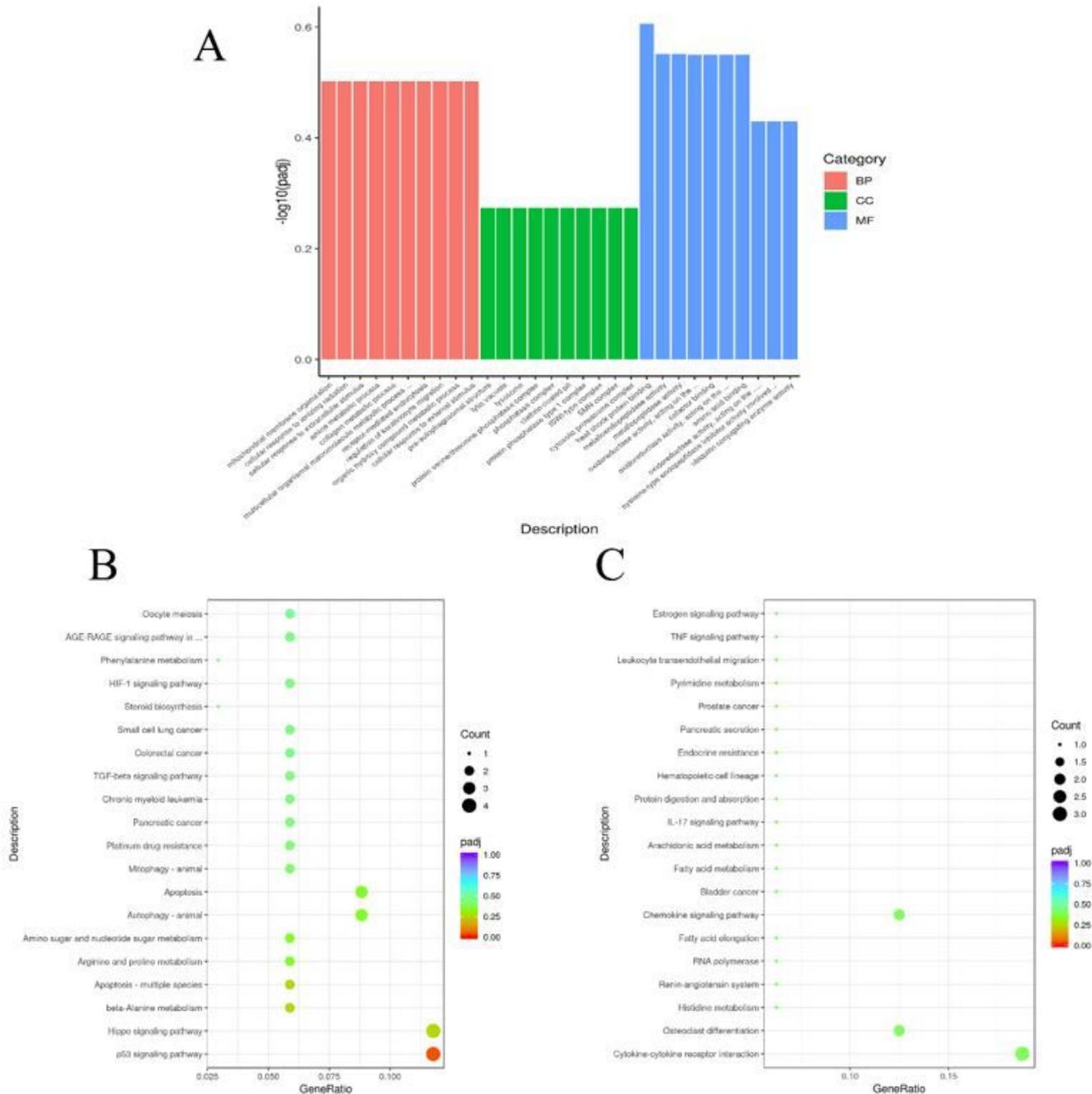


Figure 3

The differentially expressed genes of different genotypes in mice were augmented in the signaling pathway related to the small phenotype of mice spleen. (a) GO enrichment analysis histogram. In the figure, the abscissa is the GO Term, and the ordinate is the significance level of GO Term enrichment,

represented by $-\log_{10}(P_{adj})$, and different colors represent different functional classifications. (b) Rich distribution points of 20 upregulated KEGG pathways. The abscissa of the figure is the ratio of the number of differential genes annotated to the total number of differential genes on the KEGG pathway, and the ordinate is the KEGG pathway. (c) Rich distribution points of 20 down-regulated KEGG pathways.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile3sheet1.xls](#)
- [Additionalfile3sheet2.xls](#)
- [Additionalfile3sheet3.xls](#)
- [AuthorChecklist2021.1.24.pdf](#)