

RESEARCH ARTICLE

Evaluation of the mechanism underlying melatonin action in cholestatic liver disease treatment via network pharmacology, molecular docking, and in vivo experiments

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Abstract

The aim of this study was to investigate the mechanism underlying the action of melatonin (MT) in treating cholestatic liver disease. Melatonin and therapeutic targets for cholestatic liver disease were screened. A protein–protein interaction network was constructed using intersecting targets. Core targets were subjected to GO and KEGG enrichment analyses. We evaluated core target affinity through molecular docking. Biochemical indicators were measured in a mouse model of cholestasis to determine the pathological changes in liver tissue. The expression of core targets (MMP9, EGFR, and AKT) was detected through western blotting. The core targets for treating cholestatic liver disease included ALB, AKT1, ESR1, CASP3, PPARG, MMP9, PTGS2, SRC, EGFR, and IGF1. The biological processes included lipopolysaccharide stress response, bacterial molecular response, nutrient level response, and regulation of inflammatory response. Additionally, the estrogen, tumor necrosis factor-alpha, and VEGF signaling pathways were enriched in cholestatic liver disease. Molecular docking showed that MT had a strong binding affinity for MMP9, EGFR, and AKT1. Animal experiments demonstrated that melatonin alleviated inflammation and fibrosis in cholestatic liver disease, downregulated MMP9 expression, and upregulated the expression of EGFR, AKT, and phosphorylated AKT. Network pharmacology predictions suggested that these targets are closely associated with the estrogen signaling pathway. In conclusion, the protective effect of melatonin against cholestatic liver injury is likely mediated through the downregulation of MMP9 and upregulation of EGFR/AKT.

Introduction

Cholestasis-induced liver disease is a type of liver and gallbladder disease characterized by the abnormal accumulation of bile acids (BAs) in the liver due to various

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Abbreviations: SMILES, Simplified molecular-input line-entry specification; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DDC, 3,5-Diethoxycarbonyl-1,4-dihydrocollidine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBA, total bile acid; TBIL, total bilirubin.

internal and external factors, including extrahepatic and intrahepatic cholestasis [1,2]. Currently, the treatment for cholestatic liver disease mainly focuses on two aspects: nuclear receptors and their signaling pathways, particularly farnesoid X receptor and peroxisome proliferator-activated receptors, and the enterohepatic circulation of BAs [3–5]. Although significant advancements have been made in the treatment of cholestatic liver disease, some patients still experience limited efficacy or ineffective responses due to the diverse and complex pathogenesis of the condition. Therefore, the active exploration of relevant therapeutic targets is of clinical significance.

Melatonin (MT), also known as N-acetyl-5-methoxytryptamine, is a downstream metabolite of serotonin. It was first discovered in the pineal gland and has long been regarded as an indole amine related to the regulation of periodic rhythms, including day-night and seasonality [6–8]. Subsequently, researchers discovered MT in multiple peripheral tissues outside the pineal gland [9–12]. MT plays a protective role in liver diseases through various pathways, including clearing oxygen free radicals, antioxidation, inhibiting liver cell necrosis and apoptosis, thereby improving mitochondrial morphology, and inhibiting bile duct proliferation and fibrosis [13,14]. In addition, MT has been shown to prevent the occurrence and development of cholestatic liver disease [15,16]. However, the potential therapeutic targets, biological functions, and signaling pathways involved in the protective effects of MT in cholestatic liver disease remain unknown.

With rapid advances in artificial intelligence and big data, network pharmacology has become a novel method for systematically elucidating the regulatory mechanisms of drugs in diseases [17]. Therefore, in this study, we aimed to use network pharmacology combined with animal experiments to verify the potential therapeutic effects of MT in cholestatic liver disease, providing a foundation for its use in cholestatic liver disease treatment. The workflow of this study is illustrated in Fig 1.

Network pharmacology methods

Prediction of melatonin targets

The PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) is a chemical information repository that can be used to search for information on chemicals, proteins, or genes [18]. Using “melatonin” as the keyword, the canonical SMILES and 3D structure of MT were obtained from this database. The SMILES of MT was uploaded to the Swiss Target Prediction database (<http://www.swisstargetprediction.ch/>) and the species parameter was set to “Homo sapiens” to effectively predict protein targets [19]. The 3D structure of MT was uploaded to the Pharm Mapper database (<http://www.lilab-ecust.cn/pharmmapper/>), thereby identifying potential drug targets [20]. The target parameter was set to “Human Protein Targets Only”. The DrugBank database (<https://go.drugbank.com/>) contains detailed drug data and comprehensive information regarding drug targets, drug effects, and drug interactions [21]. The TCMSP platform (<https://tcmospw.com/tcmosp.php>) is useful for identifying drug target networks and drug disease networks [22]. To improve the comprehensiveness and accuracy of drug prediction, potential targets were retrieved from both the DrugBank and TCMSP databases, with the search category in TCMSP set to “Chemical name”. All collected

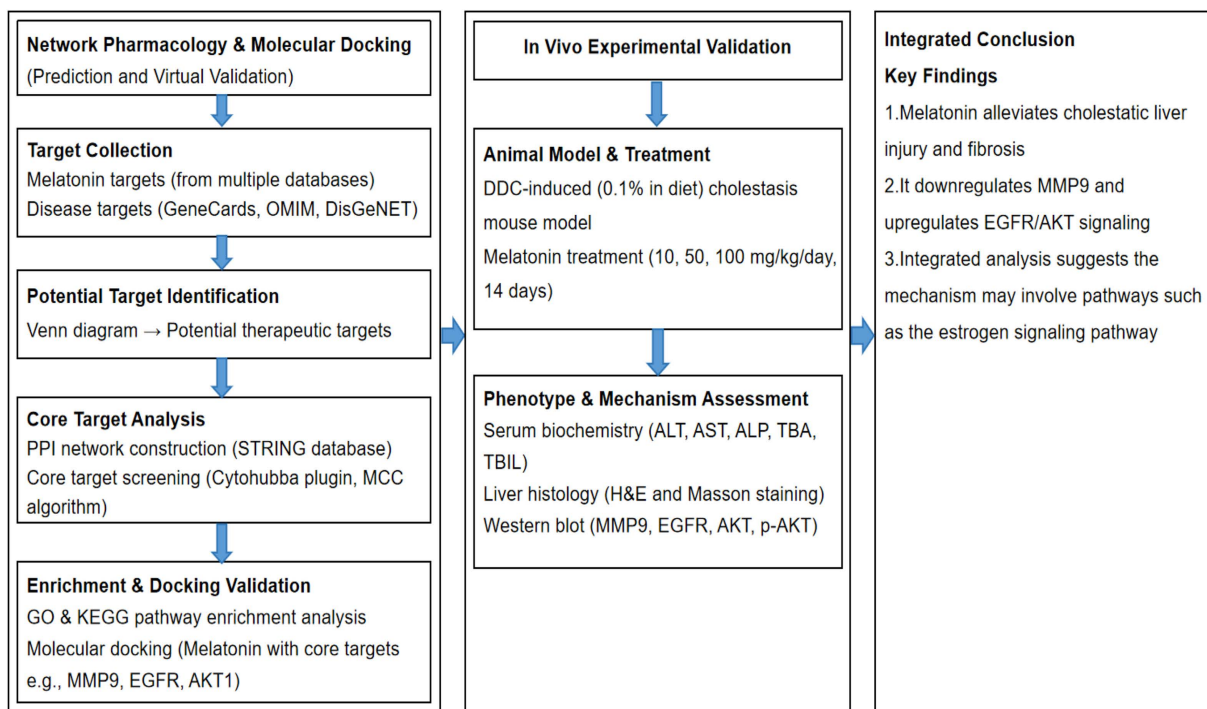


Fig 1. Workflow of this study.

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targets were then standardized using the UniProt database (<https://www.uniprot.org/>), duplicate targets were removed, and melatonin targets were obtained.

Prediction of therapeutic targets for cholestatic liver disease

GeneCards (<https://www.genecard.org/>) is a gene platform for human proteomics [23], and the OMIM database (<http://www.omim.org/>) is the primary source of continuously updated information on the relationships between human diseases and genes [24]. The DisGeNET database (<https://www.disgenet.org/>) includes data on all disease-related genes and variations in humans [25]. Using “cholestatic liver disease” as the keyword and selecting the species parameter to “Homo sapiens”, targets associated with cholestatic liver disease were screened in the aforementioned databases, with a relevance score ≥ 10 set in GeneCards and a GDA Score ≥ 0.3 set in DisGeNET. The obtained targets were summarized, duplicate targets were deleted, and the final merged targets were considered the therapeutic targets for cholestatic liver disease.

Predicting potential targets of melatonin in the treatment of cholestatic liver disease

The targets for MT and cholestatic liver disease were separately imported into the Venn diagram software (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>) to obtain potential common targets and draw a Venn diagram.

Protein–protein interaction network

We uploaded the intersecting genes to the String 12.0 database (<https://string-db.org/>) [26]. The species was set as “Homo sapiens”, a confidence threshold of >0.40 was set, and “Hide disconnected nodes” was chosen. A PPI network diagram was generated and imported into Cytoscape 3.9.1 software in tsv format. The Cytoscape plugin contained in the software was used to identify core targets using the Maximal Clique Centrality (MCC) algorithm based on degree values.

In the PPI network, darker node colors indicate higher representativeness values and a greater number of interacting targets. Core targets were selected based on the degree value.

GO functional enrichment analysis and KEGG pathway analysis

The Cluster Profiler package in R language software was used to perform GO functional enrichment analysis and KEGG pathway analysis of the intersection targets of MT and cholestatic liver disease, with a filtering condition of $P < 0.05$ [27]. The network relationship between signaling pathways and targets was visualized using the Cytoscape 3.9.1 software, and a target pathway network diagram was constructed. The green circle represents the target of the action, the blue circle the signaling pathway, and the connecting line the relationship between the target and signal.

Molecular docking of core targets

Molecular docking was performed between melatonin and the top 10 targets ranked by degree value. The crystal structure of the target protein receptor was downloaded from the PDB platform, and the protein structure was preprocessed using PyMol 2.5.5 software. Under the MMFF94 force field conditions, the 3D structure of the small molecule “Melatonin” was subjected to energy minimization processing, and the processed small molecules and receptor proteins were converted into PDBQT format using AutoDockFR 1.0 software [28]. Molecular docking was performed using AutoDock Vina 1.2.3 software [29], and the docking results were visualized using PyMol 2.5.5 software.

Materials and reagents

MT (M5250) and DDC (137030) were purchased from Sigma.

Establishment of an experimental animal model

Animal experiments in this study were approved by the Medical Ethics Committee of the Second Affiliated Hospital of Baotou Medical College (approval number: 2024-ZX-033). All experimental procedures strictly followed the rules of the Baotou Medical College Animal Experiment Center. The animal operations involved in the research process were carried out in accordance with the ARRIVE guidelines.

A total of 30 SPF-grade C57BL/6 male mice, aged 8 weeks and weighing 20–23 g, were obtained from Sibeifu Biotechnology Co., Ltd. (Beijing, China). The mice were placed in the animal room for feeding, with the indoor temperature controlled at 20–25 °C and humidity controlled at 50–55%. A 12-h alternating light/dark cycle was adopted in addition to quiet surroundings and *ad libitum* access to drinking water and feed.

Given that DDC can enhance the secretion of bile porphyrin, induce the expression of vascular cell adhesion molecules, osteopontin, and TNF- α in biliary epithelial cells, DDC-fed mice are an ideal model of bile stasis [30]. The 0.1% DDC diet was prepared by mixing DDC with chow (1:100). MT was dissolved in absolute ethanol and diluted with saline (<1% final ethanol concentration) for administration. After 1 week of adaptive feeding, the mice were randomly divided into control ($n=6$), DDC ($n=6$), low-dose DDP+MT (DDC+MT-L, $n=6$), medium-dose DDP+MT (DDC+MT-M, $n=6$), and high-dose DDP+MT (DDC+MT-H, $n=6$) groups. The control group received conventional feed, whereas the other groups received feed containing 0.1% DDC. MT was administered via intragastric gavage to the DDC+MT-L, DDC+MT-M, and DDC+MT-H groups at doses of 10, 50, and 100 mg/kg body weight per day, respectively [13,31,32]. Mice in the control and DDC groups received an equal volume of saline (ethanol concentration < 1%). The entire experimental period lasted for 14 days, with all treatments administered once daily. At the end of the modeling period, mice were fasted for 12 h prior to specimen collection.

Liver morphology, liver index, sample collection, and serum biochemical index detection

Every effort was made to minimize suffering during euthanasia. All mice were euthanized by administering an intraperitoneal injection of an overdose of 1% pentobarbital sodium (0.06 mL per 10 g body weight, approximately 60 mg/kg). This

procedure ensured rapid induction of deep anesthesia without distress. Death was confirmed by observing the cessation of heartbeat and respiration, along with the loss of corneal reflex. Blood was collected via the orbital plexus, centrifuged at 4 °C and 3000 rpm for 15 min, and the resulting serum was stored at −80 °C for subsequent analysis. Liver injury indicators such as ALT, AST, ALP, TBA, and TBIL were detected using microplate assays. The kits for the abovementioned indicators were purchased from Nanjing Jiancheng Biotechnology Co., Ltd. Mice were euthanized by inhaling CO₂ and cervical dislocation to minimize their pain as much as possible. Subsequently, the livers were separated, and their morphology was observed and photographed. Liver mass was measured, and the liver index was calculated as follows: Liver index = liver weight/body weight × 100%. A portion of the liver tissue was sectioned and fixed in 4% paraformaldehyde, whereas the remaining portion was stored in a freezer set at −80 °C.

Hematoxylin and eosin and Masson staining of liver tissue

Liver tissue was extracted from polyformaldehyde solution, and routine dehydration was performed, along with paraffin embedding, sectioning, hematoxylin and eosin staining, and Masson staining. The sections were then sealed with medium gum and viewed under a microscope to determine the pathological changes and differences in the animals.

Western blot

An appropriate amount of liver tissue was placed in lysis buffer for lysis and subsequent homogenization, followed by centrifugation for 15 min at 12,000 rpm. The protein concentration in the supernatant was measured using the BCA method; the protein sample and loading buffer were mixed at a ratio of 1:4 and boiled at 100 °C for 10 min to denature the protein. Following this, 20 µg of denatured protein sample were collected for SDS-PAGE separation. The protein bands were transferred to a PVDF membrane, a relevant primary antibody was diluted according to the manufacturer's instructions, and the PVDF membrane was incubated in the diluted primary antibody overnight at 4 °C. The membrane was subsequently incubated with the corresponding secondary antibody. Chemiluminescent staining was performed, and the membrane was imaged using a fully automated imaging analysis system. GAPDH was used as a reference. The following primary antibodies were used for these studies: MMP9 antibody (1:1000; Abcam; ab228402), EGFR antibody (1:1000; Proteintech; 30847–1-AP), AKT (1:1000; Proteintech; 10176–2-AP), P-AKT (1:1000; Proteintech; 28731–1-AP), and GAPDH (1:5000; Signalway; 52902). The band intensities of target proteins were determined using ImageJ software. All western blot experiments were independently repeated three times.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 10.0). Continuous data are presented as the mean ± standard deviation (SD). For comparisons among multiple groups, one-way ANOVA followed by Tukey's post hoc test was used if the data met the assumptions of normality and homogeneity of variances; otherwise, the non-parametric Kruskal–Wallis test was applied, followed by Dunn's post hoc test for pairwise comparisons. A *P* value of less than 0.05 was considered statistically significant.

Results

Target and network visualization of the interaction between melatonin and cholestatic liver disease

The MT targets and cholestatic liver disease targets collected from various databases were merged and deduplicated to obtain 374 MT targets and 1550 cholestatic liver disease targets. These targets were imported into Venny 2.1.0, and a Venn diagram (Fig 2A) was constructed, depicting 96 potential targets of MT for the treatment of cholestatic liver disease (S1Table). After importing these targets into the String platform, a protein–protein interaction (PPI) network was obtained (Fig 2B), showing 95 nodes and 110 edges with an average node degree of 2.32.

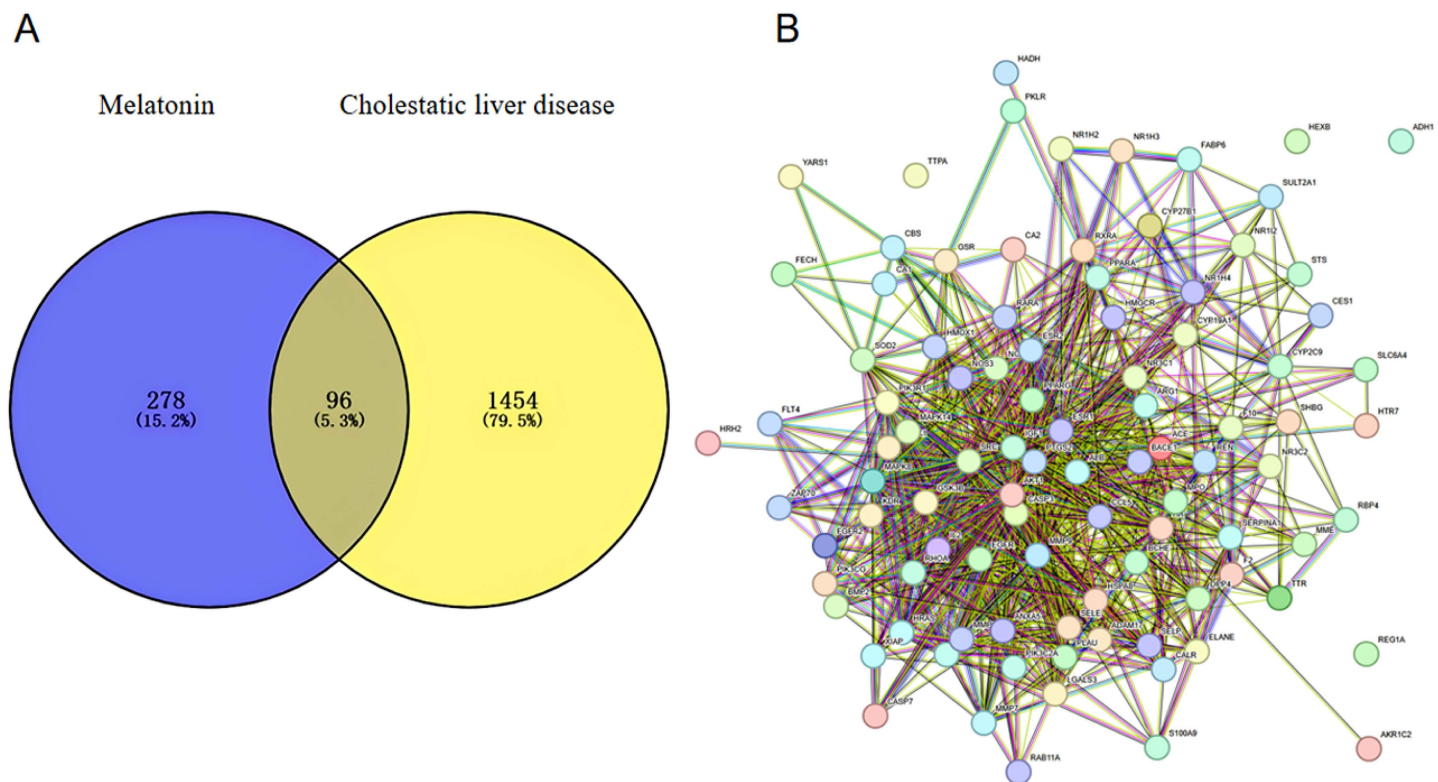


Fig 2. Identification and interaction of potential targets. (A) Venn diagram showing the 96 overlapping targets between melatonin and cholestatic liver disease. (B) Protein-protein interaction (PPI) network of the overlapping targets, with nodes representing proteins and edges representing interactions.

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Topological analysis of the PPI network was performed using Cytoscape 3.9.1 software, and the CytoHub plugin was used to visualize the drug–target–disease network (Fig 3). In the network, nodes represent target proteins, and edges represent interactions between them. Ranked in descending order by their target degree values, the top 10 core targets were identified as ALB, AKT1, ESR1, CASP3, PPARG, MMP9, PTGS2, SRC, EGFR, and IGF1.

GO and KEGG pathway enrichment analysis

GO enrichment analysis identified 1897 entries that were classified into biological processes (BP, $n = 1697$), cell composition (CC, $n = 52$), and molecular function (MF, $n = 148$). The top 10 items with the lowest P values in each category were visualized and analyzed, and a GO enrichment analysis bar chart was drawn (Fig 4A). The BPs mainly involved response to lipopolysaccharide, response to molecules of bacterial origin, response to nutrient levels, intracellular receptor signaling pathway, and regulation of inflammatory response. CCs included membrane raft, membrane microdomain, membrane region, vesicle lumen, and cytoplasmic vesicle lumen. MFs included nuclear receptor activity, ligand-activated transcription factor activity, steroid binding, phosphatase binding, and carboxylic acid binding.

Subsequently, KEGG enrichment analysis identified 144 signaling pathways using the KEGG database [33–35], with the main enriched pathways including Lipid and atherosclerosis (hsa05417), tumor necrosis factor (TNF) signaling pathway (hsa04668), EGFR tyrosine kinase inhibitor resistance (hsa01521), VEGF signaling pathway (hsa04370), Estrogen signaling pathway (hsa04915), and Focal adhesion (hsa04510) (Fig 4B) (S2 Table).

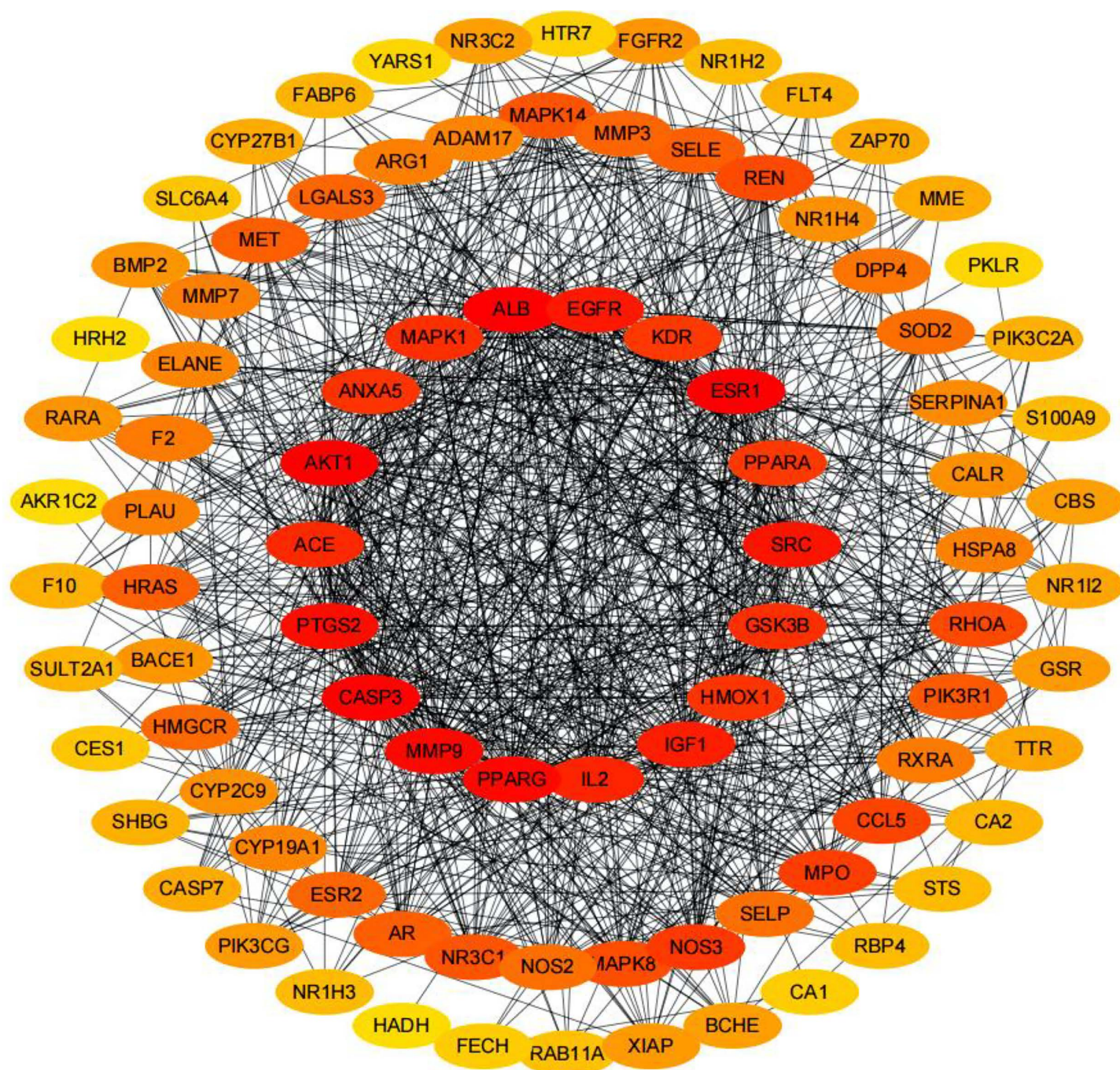


Fig 3. Topological analysis of targets for melatonin in treating cholestatic liver disease. In the network, nodes represent potential targets of melatonin for treating cholestatic liver disease. A higher degree value is indicated by a darker node color (red), denoting greater importance of the target in the therapeutic mechanism.

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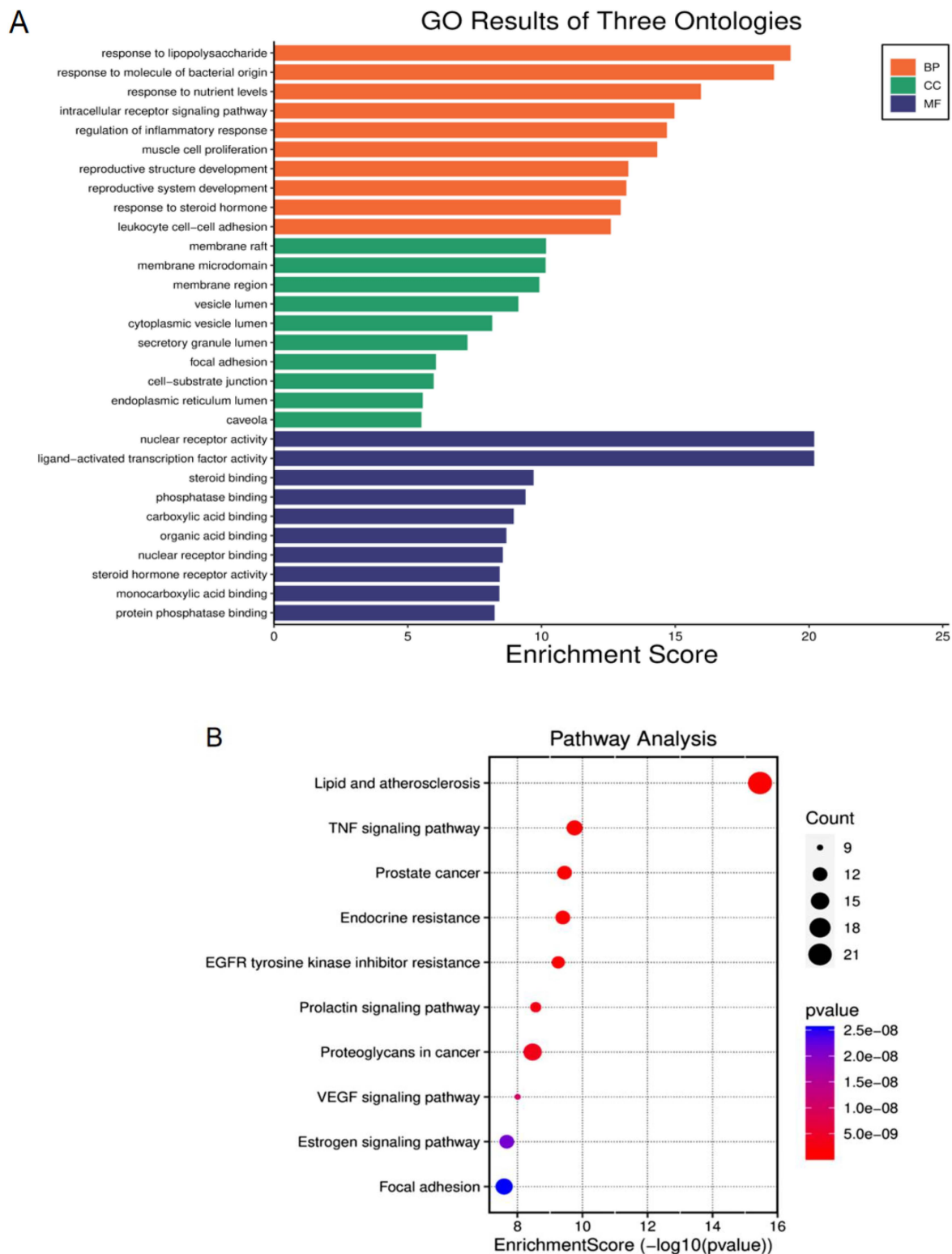


Fig 4. GO and KEGG enrichment analysis of 96 potential targets of melatonin against cholestatic liver disease. (A) Top 10 significantly enriched terms in Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) categories of Gene Ontology (GO). (B) Bubble plot of the top 10 enriched KEGG pathways. The bubble size represents the number of target genes, and the color indicates the significance level ($-\log_{10}(P\text{-value})$).

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Fig 5 shows the network of the relationships between the signaling pathways and targets, indicating that MT plays a role in the treatment of cholestatic liver disease through these signaling pathways and targets.

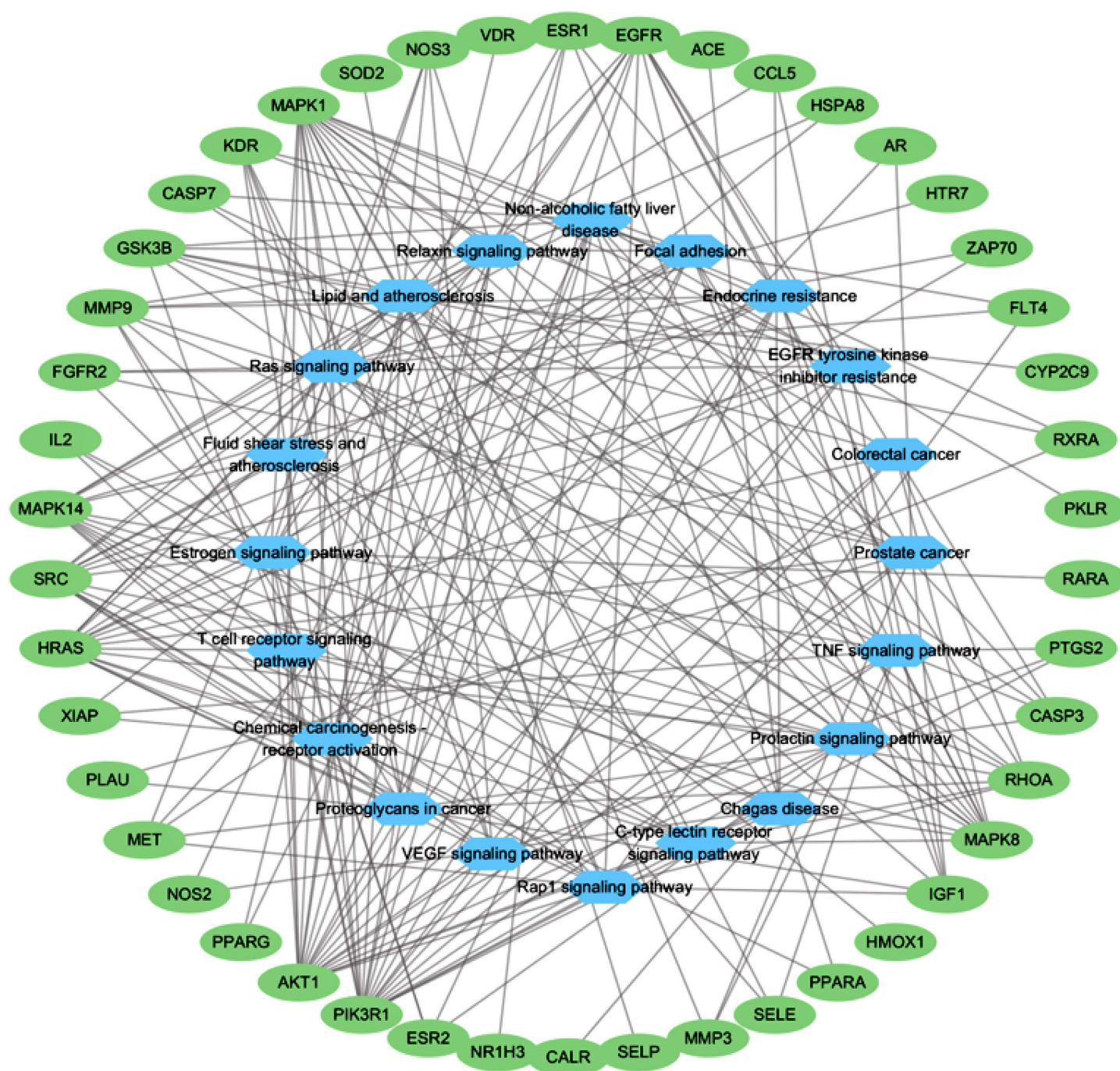


Fig 5. Target-pathway network of melatonin for treating cholestatic liver disease. Green circles denote the key targets of melatonin for treating cholestatic liver disease, blue circles represent the key signaling pathways involved, and each edge indicates a target-pathway association.

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Molecular docking

Molecular docking demonstrated a negative intermolecular binding force, indicative of the possibility of binding. The lower the binding energy, the stronger the intermolecular affinity. A binding energy < -5 kcal/mol indicates a high degree of binding between the active ingredient of the drug and the core target [36]. Fig 6 (A–J) (S1 Fig) shows that the binding affinity between the top 10 core targets and MT was < -5.0 kcal/mol (Table 1), indicating that these targets bind well with MT, suggesting their potential importance for treating cholestatic liver disease.

Effect of melatonin on liver morphology and liver index

After intervention, mouse livers were collected for observation (Fig 7A). The livers of the control mice had a pink appearance, smooth surface, and soft texture, whereas those of mice in the DDC group had a dark black appearance, rough surface, and granular texture. However, the livers of mice in the DDC + MT-L, DDC + MT-M, and DDC + MT-H groups had a brownish-red appearance and a frosted feel upon touch compared to those in the DDC group. Compared with the control group, the body mass of mice in the DDC group decreased and the liver index value increased. Contrarily, compared with the DDC group, the body mass of mice in each dose group of MT increased, and the liver index values showed varying degrees of decrease (Fig 7B, C) (S3 Table). These results indicate that MT alleviates cholestatic hepatomegaly.

Melatonin improves cholestatic liver injury and fibrosis

HE staining confirmed infiltration of the hepatic portal area by many inflammatory cells in DDC group mice, along with a large amount of bile porphyrin deposition in the bile duct (Fig 8A, B). Additionally, fibrosis and collagen deposition were observed around the bile duct. In contrast, mouse livers in the DDC + MT-L, DDC + MT-M, and DDC + MT-H groups exhibited significantly improved liver inflammation and cholestasis compared to those in the DDC group. Similarly, Masson staining showed that fibrosis in the DDC + MT-L, DDC + MT-M, and DDC + MT-H groups decreased to varying degrees compared to that in the DDC group (Fig 8C, D) (S4 Table). These results indicate that MT can effectively improve liver injury and fibrosis caused by cholestasis.

Melatonin improves liver biochemical indicators

The detection results of the microplate method are shown in Fig 8E–I. Mice in the DDC group exhibited significantly increased serum ALT and AST levels compared to those in the control group. Similarly, mice in the DDC group showed significantly increased levels of ALP, TBA, and TBIL compared to control mice. However, mice in the DDC + MT-L, DDC + MT-M, and DDC + MT-H groups exhibited significantly reduced serum ALT and AST levels and ALP, TBA, and TBIL levels compared to those in the DDC group (S4 Table). These results indicate that MT can effectively protect against liver damage caused by bile stasis.

Melatonin downregulates MMP9 and upregulates EGFR/AKT expression

Molecular docking results identified MMP9 as the core target with the strongest affinity for MT. Previous research has shown that MT alleviates bile stasis through the PI3K/AKT signaling pathway [37]. Combined with the examination of the estrogen signaling pathway (Fig 9), the results suggest that EGFR is an upstream target of the PI3K/AKT signaling pathway and a downstream target of MMP9.

Additionally, network pharmacology revealed that MMP9, EGFR, and AKT are important potential targets for the treatment of cholestatic liver disease. Our results (Fig 10. A–E) showed that the expression level of MMP9 in the liver tissue of DDC group mice was the most significant. After oral administration of different doses of MT, the expression level of MMP9 decreased to varying degrees. After oral administration of different doses of MT to mice in the DDC group, the expression levels of EGFR, AKT, and P-AKT in liver tissue were significantly increased. These results suggest that MT may downregulate MMP9 and upregulate EGFR/AKT expression (S5 Table).

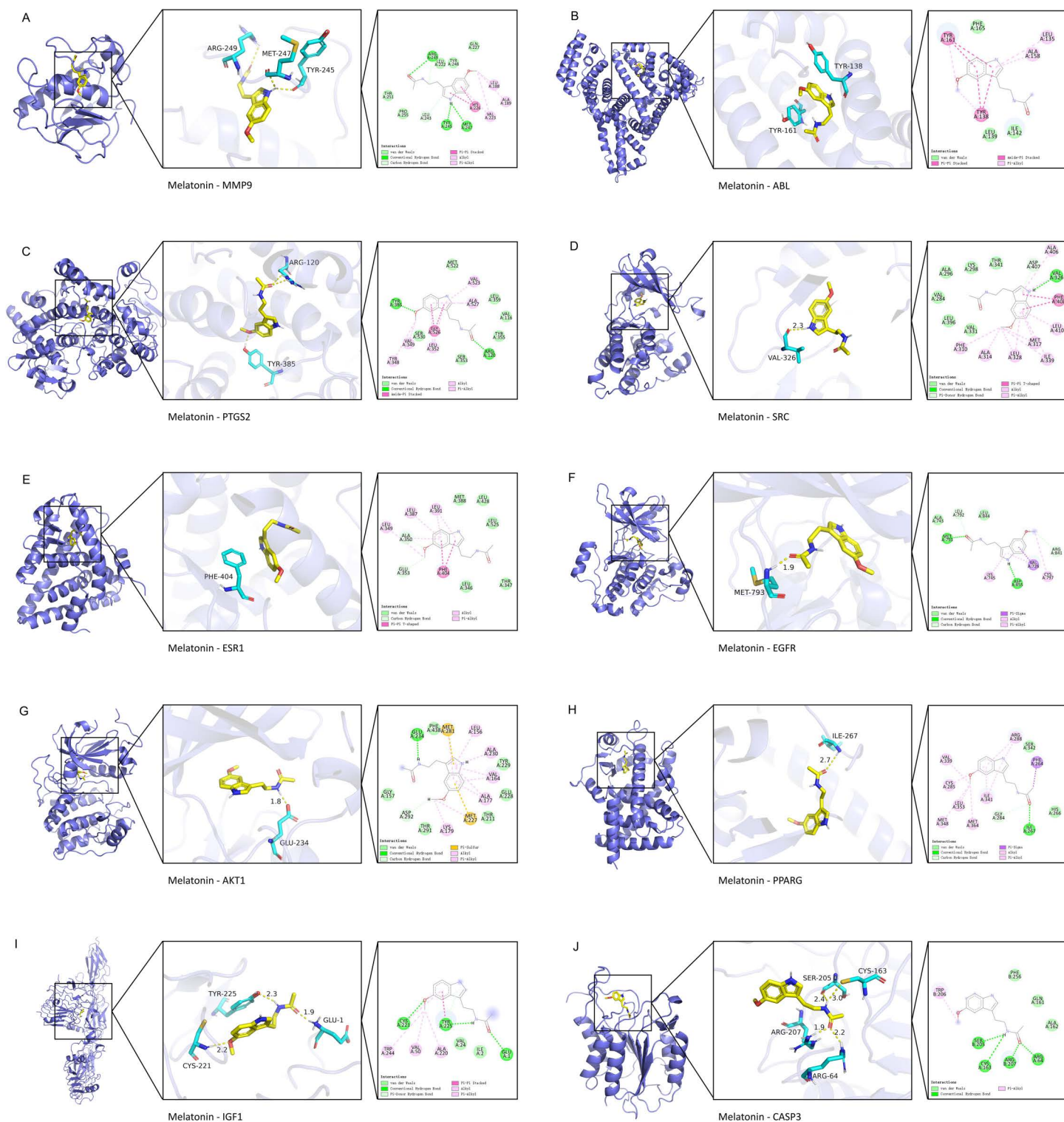


Fig 6. Molecular docking of melatonin with the top 10 core targets. Binding modes of melatonin to the top 10 core targets (A-J: MMP9, ALB, PTGS2, SRC, ESR1, EGFR, AKT1, PPARG, IGF1, and CASP3). The left panel shows the overall view, with the protein structure colored in blue. The middle panel provides a detailed close-up view, where melatonin is shown in yellow, the interacting protein amino acid residues are colored in cyan, and yellow dashed lines indicate hydrogen bonds. The right panel displays the 2D ligand-interaction diagram, with pink circles denoting hydrophobic interactions.

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Table 1. Molecular docking results of melatonin with core targets.

Core target receptor	Protein structure ID	Binding site amino acid	Binding energy/ (kJ·mol ⁻¹)	Degree
ALB	7JWN	TYR-161, TYR-138	-8.378	71
AKT1	GV1	GLU-234	-6.905	61
ESR1	2YJA	PHE-404	-6.964	54
CASP3	3DEK	ARG-207, SER-205, CYS-163, ARG-64	-5.647	53
PPARG	2ZK0	ILE-267	-6.518	51
MMP9	6ESM	ARG-249, MET-247, TYR-245	-8.472	51
PTGS2	5IKR	TYR-385, ARG-120	-7.518	50
SRC	7NG7	VAL-326	-7.21	49
EGFR	4I22	MET-793, ASP-855	-6.956	46
IGF1	5u8r	TYR-225, CYS-221, GLU-1	-6.207	45

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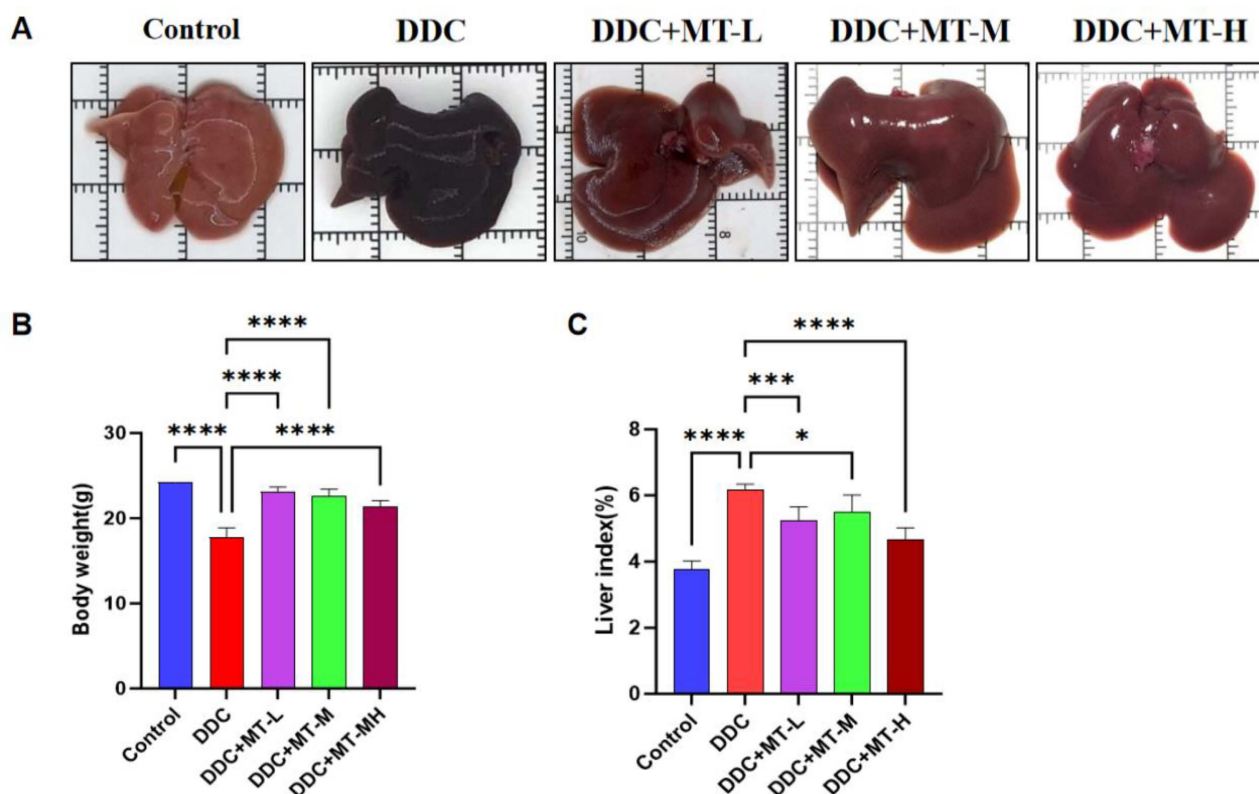


Fig 7. Liver morphology, body weight, and liver index across experimental groups. (A) Gross appearance of liver tissue from mice in each group following intervention with different concentrations of melatonin. (B) Changes in body weight of mice in each group after intervention with different concentrations of melatonin. (C) Changes in liver index (liver weight/body weight × 100%) of mice in each group after intervention with different concentrations of melatonin. Data are presented as mean ± SD (n=6). Significant differences compared to the DDC group are indicated (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

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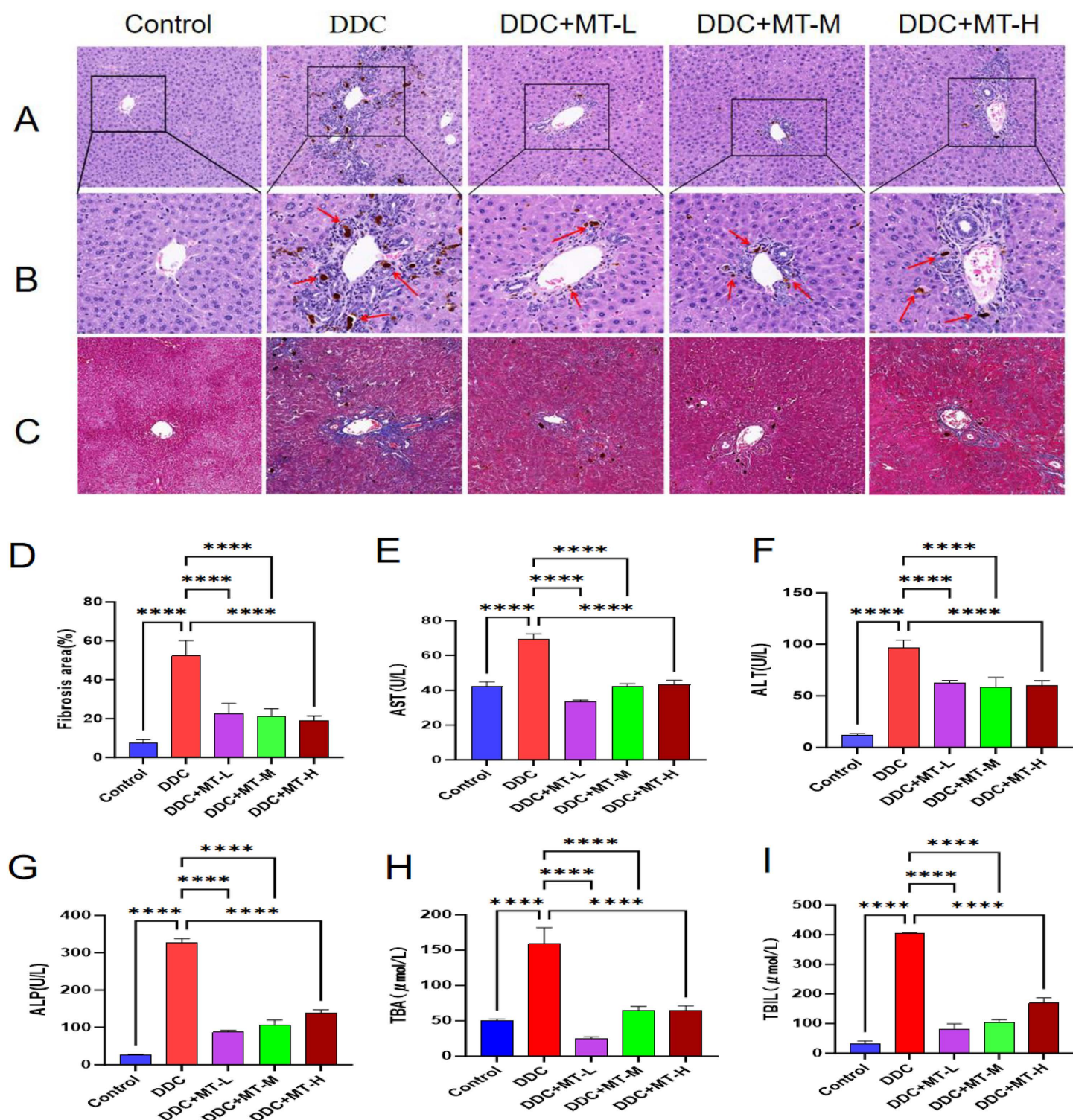


Fig 8. Melatonin ameliorates liver injury, fibrosis, and serum biomarkers in cholestatic mice. (A) Representative hematoxylin and eosin (H&E)-stained liver sections from mice in each group at 100 $\times$ magnification. (B) Representative H&E-stained liver sections at 200 $\times$ magnification; red arrows indicate bile porphyrin deposits. (C) Representative Masson-stained liver sections from each group ($\times$ 100), where blue color indicates collagen fibers. (D) Quantitative analysis of the collagen fiber area percentage in liver tissues. (E–I) Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bile acid (TBA), and total bilirubin (TBIL) in each group. Data are presented as the mean $\pm$ SD (n=6). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

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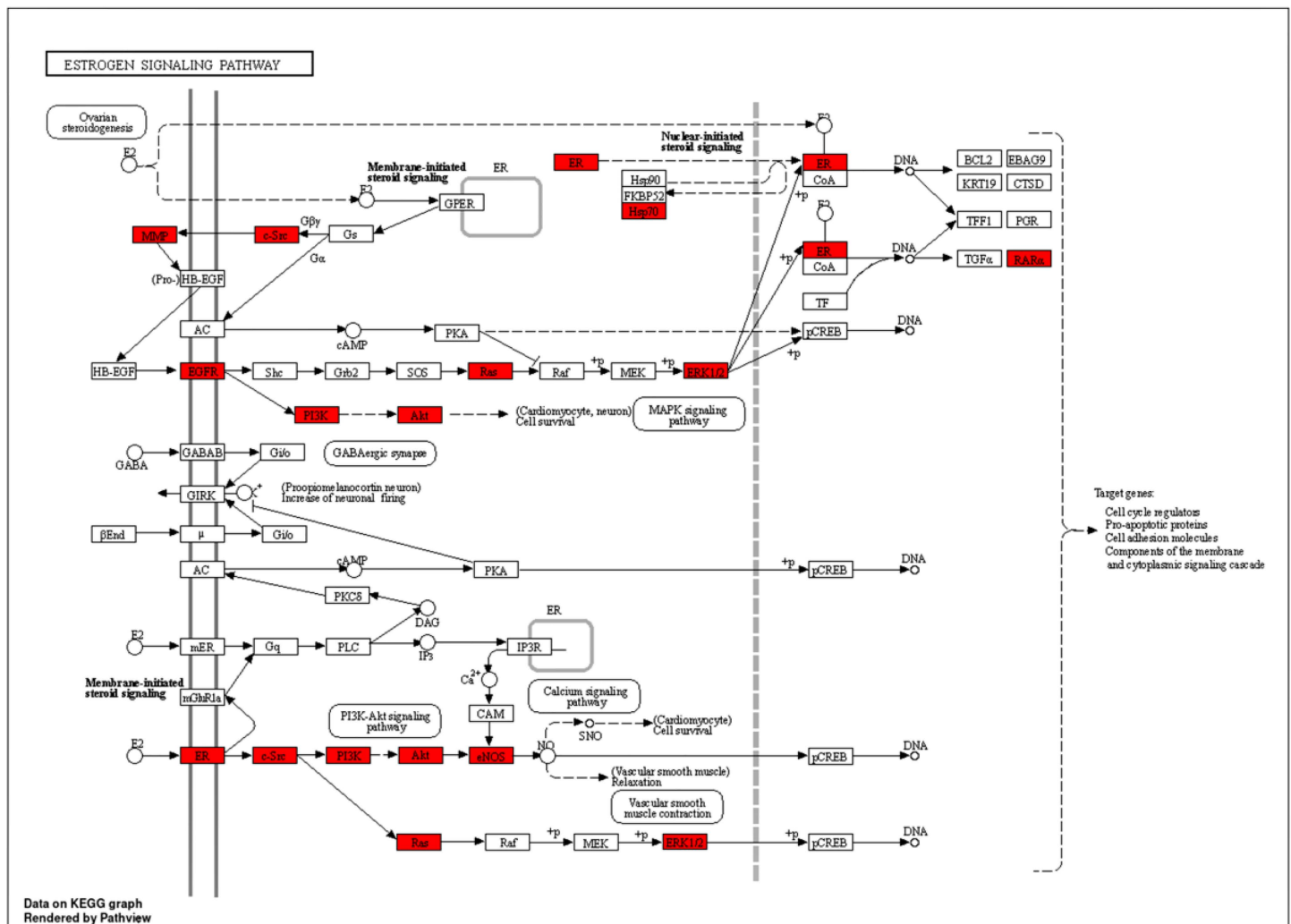


Fig 9. Distribution of Targets in the Estrogen Signaling Pathway.

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Discussion

Cholestatic liver injury is characterized by parenchymal cell death, bile duct hyperplasia, liver inflammation, and fibrosis [38]. Among these features, inflammation plays a significant role in chronic liver damage associated with the disease [39]. The invasion of inflammatory cells in cholestatic liver diseases, such as primary biliary cholangitis and primary sclerosing cholangitis, can promote the death of bile duct cells, leading to bile duct blockage and obstruction of BA excretion. The accumulated BAs can trigger inflammation and fibrosis, leading to a vicious cycle [40]. Therefore, improving the prognosis of cholestatic liver injury and delaying its progression to liver fibrosis by inhibiting inflammation has become a research focus in recent years [41]. In the present study, we explored and validated the potential mechanism by which MT improves cholestatic liver disease using network pharmacology and in vivo experiments.

GO functional enrichment analysis showed that the main BPs involving the 96 targets of MT in the treatment of cholestatic liver disease were lipopolysaccharide stress response, bacterial molecular response, nutritional level response, and regulation of inflammatory response. Lipopolysaccharides bind to Toll-like receptors in bile duct epithelial cells, resulting

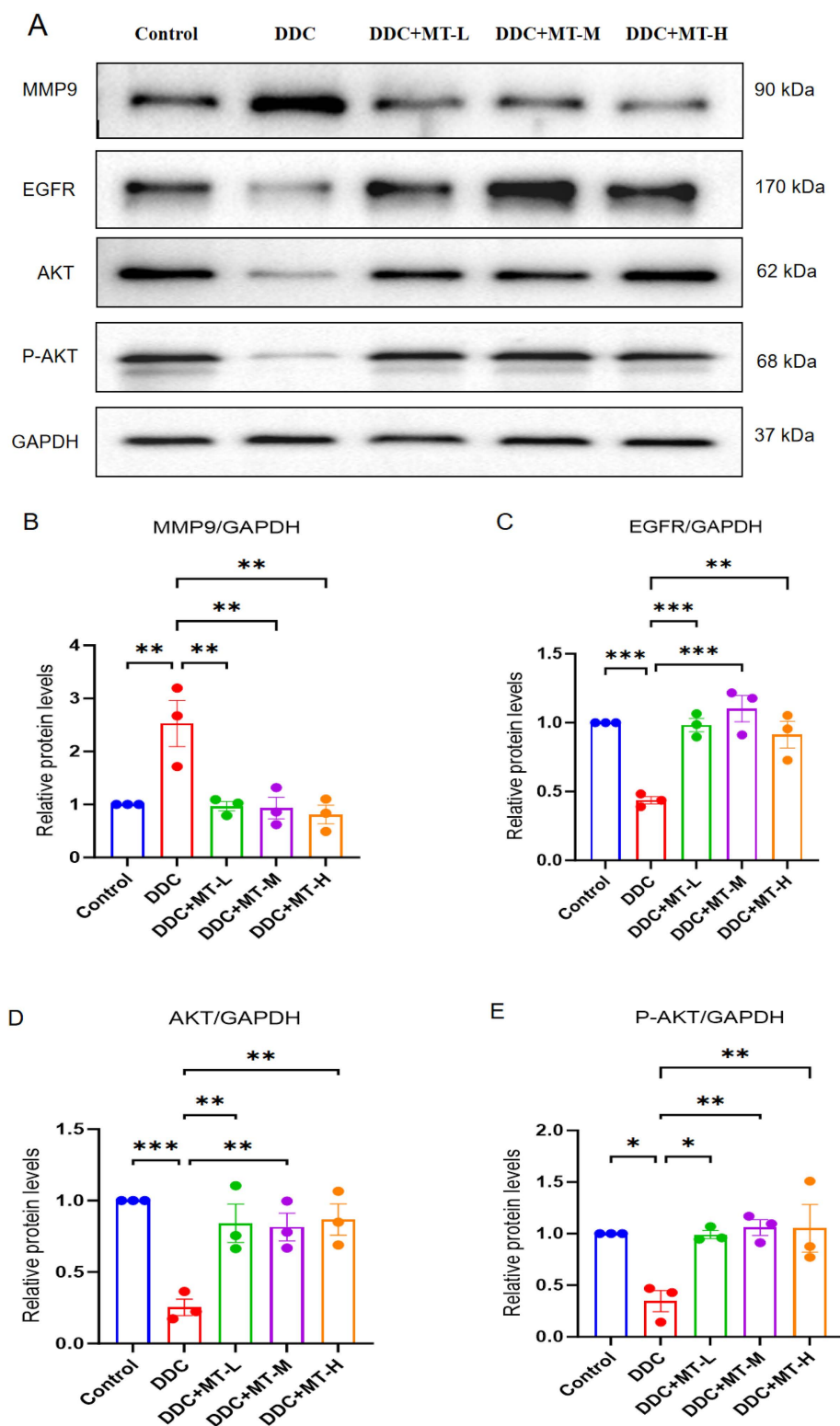


Fig 10. Western blot analysis of key protein expression in liver tissues. (A) Representative Western blot images of MMP9, EGFR, AKT, and p-AKT. (B–E) Quantitative analysis of the relative protein expression levels of MMP9, EGFR, AKT, and p-AKT, normalized to GAPDH (n=3). Data are presented as mean ± SD. Significant differences compared to the DDC group are indicated (*P<0.05, **P<0.01, ***P<0.001).

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in an inflammatory cascade reaction, which causes liver inflammation and fibrosis [42]. Subsequently, immune cells that infiltrate after liver injury secrete proinflammatory cytokines, promoting a cascade reaction in liver fibrosis [43]. In this regard, MT reduces the expression of inflammatory factors stimulated by BAs *via* inhibiting the ERK/Egr1 signaling pathway, thereby alleviating cholestatic liver injury [31]. Accordingly, through KEGG pathway analysis, we found that the main signaling pathways through which MT may alleviate cholestatic liver disease include TNF, VEGF, and estrogen signaling pathways. TNF and its receptor pathways induce hepatocyte apoptosis by activating caspases [44]. Thus, necrotic apoptosis of liver cells may be a mechanism underlying cholestatic liver injury, mediated by the TNF- α signaling pathway. Relevant markers (RIPK1, RIPK3, and MLKL) have been identified in the livers of patients with primary biliary cholangitis (PBC) and mice with bile duct ligation (BDL). Moreover, necrotic cell death triggers inflammation by releasing damage-associated molecular patterns into the extracellular space [38]. Thus, TNF- α is a key pro-inflammatory and pro-fibrotic cytokine driving liver fibrosis and exerts its anti-fibrotic effects by reducing glutathione and inhibiting the secretion of procollagen alpha 1 [45]. The VEGF signaling pathway is also closely associated with cholestatic liver injury [46,47]. Chlorigenic acid effectively improves cholestatic liver injury in rats by inhibiting VEGF [48]. Moreover, the liver is one of the target organs for sex hormones. For instance, estrogen regulates the proliferation and secretion of bile duct cells through receptors, thereby affecting the progression of PBC [49]. Particularly, estrogen can prevent nuclear translocation of NF- κ B *via* ESR1 to suppress inflammatory gene expression [50] and actively regulate the growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis [51]. IGF1 regulates the proliferation of bile duct cells and improves energy metabolism [52,53]. However, estrogen can also inhibit BA secretion *via* the ESR1/FXR/BSEP pathway, leading to intrahepatic bile stasis [54]. In this regard, Dong et al. reported that activation of the PI3K/AKT and MAPK signaling pathways is an important mechanism in estrogen- and cholestasis-induced liver injury [55].

Our network pharmacology approach predicted a potential role for the estrogen signaling pathway in the treatment of cholestatic liver disease using MT. Further analysis of the target-pathway network (Fig 5) identified MMP9, EGFR, and AKT1 as pivotal targets in this pathway. The molecular docking results verified strong binding between MT and all these targets, particularly MMP9, which showed the highest affinity. MMP9 is a gelatinase and a member of the matrix metalloproteinase (MMP) family. Liver fibrosis is a common pathway involved in the development of various chronic liver diseases, including cholestatic liver disease. MMPs play an important role in the formation and regeneration of liver fibrosis owing to their effects on cell proliferation, gene expression, and apoptosis, making them therapeutic targets for chronic liver diseases [56]. MMP9 participates in the degradation of type IV collagen, fibronectin, and elastin, thereby promoting liver fibrosis and scar regression [57]. In the context of cholestatic liver disease, EGFR activation exhibits protective and anti-fibrotic effects against cholestatic liver injury [58]. In addition, the EGFR ligand, heparin-binding epidermal growth factor, plays a protective role in cholestatic liver fibrosis [59]. Furthermore, AKT is a key regulatory kinase that is activated by the phosphorylation of two key regulatory sites, *via*. Thr308 and Ser473. It transmits signals through the PI3K/Akt cell signaling cascade, which regulates cell survival and apoptosis [60]. Accordingly, Li et al. found that MT activates Nrf2 through the PI3K/Akt-dependent pathway to alleviate oxidative stress and improve ANIT- and cholestasis-induced liver injury in rats; AKT itself possesses anti-apoptotic properties [37]. In this study, we validated the relevant targets of MT in cholestatic liver disease by constructing a mouse cholestasis model. Our findings demonstrated that MT treatment significantly downregulated MMP9 and upregulated EGFR/AKT expression, suggesting that the hepatoprotective effect of MT is mediated, at least in part, through the regulation of these key downstream effectors (MMP9, EGFR, AKT), potentially *via* modulating critical nodes within the estrogen signaling pathway.

Serum ALT and AST are commonly used indicators for detecting liver injury [61], whereas serum ALP, TBA, and TBIL can be used to assess the severity of cholestatic liver injury [62]. Our results showed that serum levels of ALT, AST, ALP, TBA, and TBIL in DDC mice were significantly higher than those in the control group. However, after oral administration of MT, the levels of these liver function biochemical indicators significantly decreased. Additionally, HE and Masson staining results showed a significant improvement in liver tissue inflammation and fibrosis post MT administration. These findings

demonstrate the protective effect of MT against DDC-induced cholestatic liver injury and suggest its potential as a therapeutic candidate.

This study has some limitations. First, no standard positive control drug (e.g., ursodeoxycholic acid, UDCA) was included in the experimental design. Future studies should consider incorporating a positive control to enable a more direct evaluation of the potential advantages or distinctive features of MT relative to standard therapy under identical experimental conditions. Second, the mechanistic conclusions are primarily derived from observed changes in protein expression. Future work should employ gain- and loss-of-function experiments (e.g., using specific inhibitors or gene knockdown) to establish a causal relationship. Furthermore, direct detection of upstream signaling molecules (such as estrogen receptors) remains to be performed. Future research should, based on an expanded sample size, further explore the dose-response relationship and combine in vitro experiments with validation of upstream targets to provide a more in-depth foundation for the therapeutic use of MT in cholestatic liver disease.

In summary, this study predicted multiple core targets of MT for the treatment of cholestatic liver disease using network pharmacology. We established an animal model of bile stasis, in turn confirming that MT alleviates cholestatic liver injury, and that its mechanism of action may be related to the downregulation of MMP9 and upregulation of EGFR/AKT. Notably, the relevant biochemical indicators and protein expressions did not show a sustained increase or decrease with changes in MT concentration, which may be related to the complex metabolic dynamics of MT in the body.

Supporting information

S1 File. Raw images.

(DOCX)

S1 Fig. Molecular docking of melatonin with the top 10 core targets.

(PDF)

S1 Table. The list of 96 overlapping targets.

(XLSX)

S2 Table. Complete list of enriched KEGG pathways.

(XLSX)

S3 Table. Raw data for body weight and liver index measurements.

(XLSX)

S4 Table. Raw data for serum biochemical indicators.

(XLSX)

S5 Table. Raw densitometry data for western blot analysis.

(XLSX)

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Investigation: Tao Li.

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Project administration: Wang Jing.

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Software: Tao Li.

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Validation: Tao Li.

Visualization: Tao Li.

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