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RESEARCH ARTICLE

Molecular Mechanism Network Pharmacology and Bioinformatics Research of Qingrejiedu Decoction in Treatment of Liver and Gallbladder Neoplasms

Yangyi Zhang

Fujian University of Traditional Chinese Medicine
*Corresponding author: m13860992725@163.com

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ABSTRACT

The Chinese Qingrejiedu Decoction (QDD) is a traditional medicine prepared from some herbs. QDD has the potential to treat and manage liver and gallbladder neoplasms. Our main objective is to examine the molecular mechanism network pharmacology and perform bioinformatics research on QDD for the treatment of liver and gallbladder neoplasms. We used Gene Expression Omnibus (GEO) database to obtain GSE106671 expression profiling dataset by array type based on GPL16956 platform. The dataset comprised liver metastatic invasion and metastasis of gallbladder cancers; there were three samples of parental gallbladder cancer cells and three samples of highly metastatic gallbladder cancer cells. We used the Traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP) to identify targets of QDD in liver and gallbladder neoplasms. All the Protein-protein interaction (PPI) networks were analyzed in STRING and Cytoscape R software. Optimal animal model was determined by Multi-Ontology Enrichment Tool (MOET) enrichment. Our study identified 113 significant differentially expressed genes (DEGs), including 47 up-regulated and 66 down-regulated genes. The PPI network analysis revealed ten hub genes that were essential in the progression of liver and gallbladder neoplasms. The KEGG pathways were enriched in C-type lectin receptor signalling (CLR), Vascular endothelial growth factor (VEGF) signalling, P53 signalling, and nucleotide-binding oligomerization domain (NOD)-like receptor signalling, while the biological pathways were enriched in catabolism and metabolism of collagen, regulation of inflammatory response, cell death, defense response. Overall, Bonobo appears to have the strongest statistical evidence of association between the hub genes and liver cancer. QDD seems to be a significant medicine in treating and diagnosing gallbladder and liver neoplasms.

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INTRODUCTION

Qingrejiedu Decoction (QDD) is a traditional Chinese medication used to treat liver injuries (Ma *et al.*, 2021). Liver and gall bladder neoplasms refer to abnormal tissue masses generated by the overgrowth of cells and division than the recommended levels. Neoplasms could be benign or malignant. Benign neoplasms grow and become large; however, they do not invade or spread into surrounding tissues (Cullen and Popp, 2002). In contrast, malignant neoplasms spread into surrounding tissues and other body organs through the blood and lymphatic systems (Cullen and Popp, 2002).

The Chinese QDD is a traditional medicine prepared from *Citrus aurantium, Rehmannia glutinosa (Gaertn.),* and *Rheum palmatum*. Lin *et al.* (2021) proposed that Qingre refers to the heat-clearing effect while Jiedu refers to the detoxifying effect of QDD. QDD has been identified to improve clinical symptoms and reduce the effects of chronic liver and gall bladder infections (Zhang *et al.*, 2020; Ma *et al.*, 2021). Furthermore, QDD increases liver protection and reduces the absorption of endotoxins. Removing endotoxins lowers the levels of liver inflammation and reduces the rate of liver destruction.

Ai and Xie (2022) suggested that QDD can be modified and prepared using the "*Lichong decoction*" antitumor formula. The formula involves peach seeds, Mongolian milkvetch roots, tuckahoes and dandelions. These herbs are efficacious in increasing blood circulation and detoxification of the spleen. Also, it boosts the immune

response of patients. Li *et al.* (2022) suggested that QDD has antifibrosis and anti-inflammatory effects in post-myocardial infarction.

Liver carcinogenesis results in the transformation of malignant hepatocytes at the physiological level. Physiological adaptations of hepatocytes are significant indicators of malignant transformation and offer a possibility to explain the growth and development of neoplasms through chronic evolutions and cellular transformations (Lamps *et al.*, 2021; Cullen and Popp, 2002). Liver carcinogenesis involves several complex processes associated with the similarity between preneoplastic and precancerous lesions and the limited response of hepatic cells.

Gallbladder tumors can be categorized as adenoma, carcinoma and lesions. Gallbladder adenoma is characterized by nodules of varying sizes that range from 0.5 mm to 100 mm in diameter on its surface (Lamps *et al.*, 2021). These nodules are extremely shiny, highly fragile and have a reddish to yellowish surface according to the duration. Adenocarcinoma constitutes malignant tumors of the epithelium with neoplasms on larger areas of the mucosa. Papillary and mucin-producing gallbladder neoplasms are soft and appear like cauliflower over larger areas of the mucosa (Huang *et al*., 2006; Wan *et al.*, 2017). Cystic hyperplasia consists of thickened mucosa and numerous endocrine neoplasms of smaller cysts. The mucosa is usually grey-white with a diffused and thick sponge imparted by 1 mm to 3 mm cysts in the hyperplastic mucosa (Lamps *et al.*, 2021).

Our study aims to analyze the pharmacological networks and perform bioinformatics of the differentially expressed genes in QDD to treat liver and gallbladder neoplasms. Network pharmacology of QDD involves compound-target networks to establish the underlying molecular mechanisms of QDD by screening for bioactive compounds. The targets of these bioactive compounds are then mined using online databases such as the KEGG and STRING to obtain the enrichment pathways and PPI networks. Bioinformatics permits us to analyse the DEGs associated with QDD and how their expression levels regulate the action of QDD in gallbladder and liver neoplasms.

MATERIALS AND METHODS

GEO datasets: We used the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) to obtain microarray datasets associated with liver and gall bladder neoplasms and we selected mouse model using NOZ cells (GSE106671). The GSE106671 dataset consists of expression profiling by array type based on the GPL16956 platform (Agilent-045997 Arraystar human lncRNA microarray V3). The dataset comprised liver invasion and metastasis of gallbladder cancers; there were three samples of parental gallbladder cancer cells and three samples of highly metastatic gallbladder cancer cells. We converted all probes into homologous gene symbols.

Identification and analysis of DEGs: We screened the DEGs in R software (https://www.r-project.org/) based on parental gallbladder and highly metastatic gallbladder cancer cells. Six samples were analyzed from the dataset and screened them for DEGs. GEO2R tool, R software and Network Analyst were used to identify the DEGs. We normalized before adjusting for batch effects on various gene expression profiles. The experimental groups (parental or metastatic) were distinct, allowing us to visualize and identify the differentially expressed genes (DEGs). In our analysis, we removed genes without symbols and those without a single set of probes. We set an adjusted p-value ($P \leq 0.05$) to distinguish between statistically significant and non-significant genes before applying a log fold change of greater or equal to 1. Furthermore, we extracted Venn diagrams (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html#:~:text=GEO2R%20is%20an%20interactive%20web,differentially%20expressed%20across%20experimental%20conditions) to visualize the overlapping DEGs. We generated volcano plots, mean plots and heatmaps of the up and down-regulated DEGs.

Screening of active compounds: The compounds of QDD were examined in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (https://tcmsp-e.com/tcmspsearch.php?qs=herb_all_name&q=Qingrejiedu+Decoction&token=76d7cbb951af2e422866695d80ce7976) (TCMSP) consisting of pharmacological properties of every compound used in QDD. This study adopted an oral bioavailability greater than 30%, drug-likeness above 0.18 and a CACO-2 permeability of > 0.4. Potential targets of the components of QDD were extracted using an analogous technique specified in the BATMAN-TCM (http://bionet.ncpsb.org.cn/batman-tcm/). In the drug screening and evaluation, detailed information was gathered from all databases in which the ingredients of QDD were used. The known targets linked to liver and gallbladder neoplasms were screened using keywords such as "liver neoplasms, gallbladder neoplasms" on the DisGeNET Gene database (https://www.disgenet.org/). Additionally, the names of these compounds were standardized based on PubChem CIDs (https://pubchem.ncbi.nlm.nih.gov/), and the extracted targets were then applied to the UniProt database (https://www.uniprot.org/) for gene verification.

Target identification of QDD in liver and gallbladder neoplasms: An integrated approach was adopted to determine the target proteins in bioactive compounds of QDD involving the TCMSP (https://tcmsp-e.com/tcmsp.php) and SymMap (http://www.symmap.org/) databases. Furthermore, we used the Comparative Toxicogenomics Database (CTD) (https://ctdbase.org/) to identify genes associated with gallbladder and liver neoplasms. An inference score of less than 20 was set for the desired genes whose targets were then transformed into various gene symbols based on the UniProt knowledge database (https://www.uniprot.org/ help/uniprotkb). The intersection between targets of liver and gallbladder neoplasms and the targets of QDD was marked using Venn diagrams ((https://www.ncbi.nlm.nih. gov/geo/info/geo2r.html#:~:text=GEO2R%20is%20an%20interactive%20web,differentially%20expressed%20across%20experimental%20conditions). We then constructed the related target networks in Cytoscape (https://cytoscape. org/).

Network construction and analysis: Gene lists of QDD, gallbladder and liver neoplasms were submitted to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database and set the species to "Homo Sapiens" with a confidence score of equal and higher than 0.7 (https://string-db.org/cgi/about). PPI interaction networks were extracted and exported them to Cytoscape (https://cytoscape.org/). Only PPI networks of an interaction score greater than or equal to 0.7 were extracted before subsequent PPI networks. These networks were then merged to yield a single network. The resultant network was analyzed depending on the degree (the biological function of the nodes). Markov Cluster Algorithm (MCL) was used in present study by adjusting the inflation parameter to 3 with the solid lines representing the edges. PPI clusters were built using Cytoscape.

Pathway enrichment analysis: ClusterProfiler package was adopted in R software (https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) and ShinyGO (http://bioinformatics.sdstate.edu/go/) to identify the biological pathways of QDD in liver and gallbladder neoplasms. In ShinyGO, the minimum pathway size was set as two and the maximum as 2000. Also, redundancies were removed and set a false discovery rate (FDR) cut-off of 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) was used in identifying the biological pathways of core targets. The p-value was adjusted to less than 0.05 and considered statistically significant.

Disease enrichment analysis to determine animal model for study: MOET enrichment was used to determine most optimal animal model of liver and gallbladder neoplasms (https://rgd.mcw.edu/rgdweb/enrichment/analysis.html). Ten hub genes (JUN, PTEN, VEGFA, TP53, AKT1, MYC, BCL2L1, CDKN1A, MMP9, and EGFR) were searched in the Disease Ontology for liver cancer (DOID:3571).

RESULTS

Identification and analysis of DEGs: The GSE106671 dataset consisted of 6 samples including different liver invasion metastasis of gallbladder cancer cells. The dataset used a mouse model consisting of NOZ cells in two rounds of intrasplenic injections. A highly metastatic subclone of NOZ cells was acquired and labelled as LiM2-NOZ. The long non-coding RNAs (lncRNAs) and mRNAs were also acquired in LiM2-NOZ and NOZ cells. Overall, there were 3 NOZ and 3 LiM2-NOZ cells. Nearly 113 significant DEGs were identified, including up-regulated and down-regulated genes. We fined the significance values to have an adjusted p-value of lower than 0.05 and a log2 fold change of lower than 1.0. Results showed 66 down-regulated DEGs and 47 up-regulated DEGs.

The boxplots (Fig. 1) revealed that the gene profiles were normalized using a log2 transformation. The median-centered values indicate that the normalization was effective and cross-comparisons were possible. The x-axis shows the genes and the y-axis shows their expression profiles. The boxplot shows the distribution of the gene expression values in parental GBC and highly metastatic GBC.

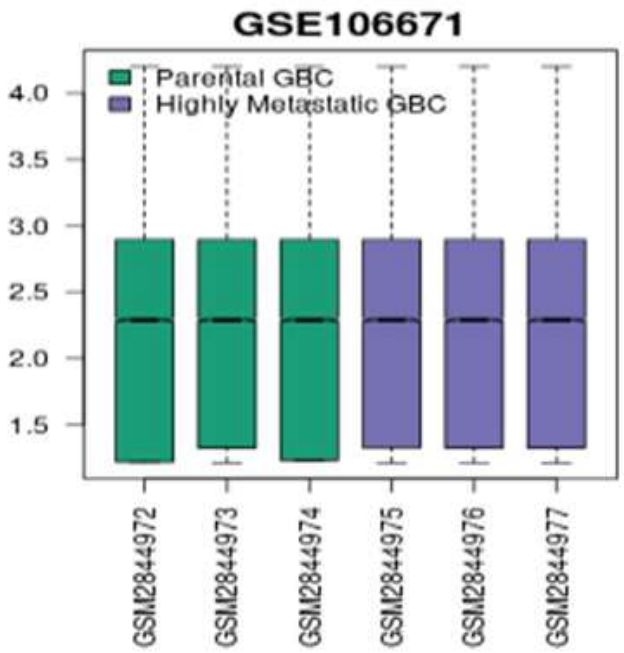

Fig. 1: Normalized gene expression profiles.

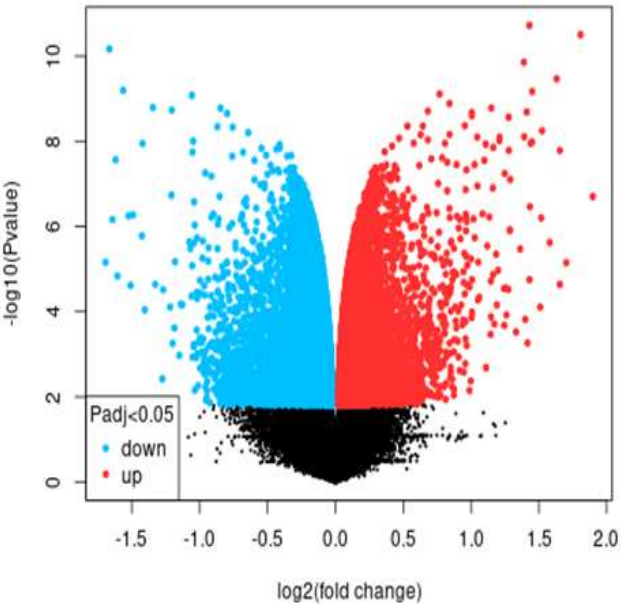

Fig. 2: Volcano plot showing the down-regulated and up-regulated genes.

Volcano plot: The volcano plot (Fig. 2) was produced using the limma package in R. It is a representation of DEGs based on the statistical significance (-log10 p-value) against the log2 fold change (change magnitude). Log2 fold change defines the ratio between transcript expression values and the logarithm of the genes. Every point on the volcano plot represents a gene annotation. The highlighted genes in red and blue are differentially expressed based on a p-value of 0.05 (red represents up-regulated DEGs, and blue represents down-regulated DEGs). The horizontal dashed line shows the statistical significance of DEGs based on the adjusted p-value. The up-regulated genes were those whose expression increased in parental GBC samples compared to highly metastatic GBC cells. In contrast, the down-regulated genes represent those genes whose expression profiles were reduced in parental GBC cells compared to highly metastatic GBC cells.

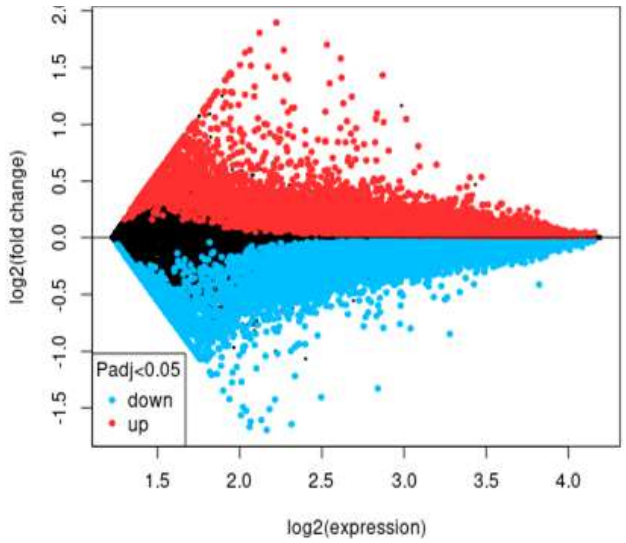

Fig. 3: Mean difference plot of differentially expressed genes.

Mean difference plot: The mean difference plot (Fig. 3) shows the average log2 expression against the log2 fold change of DEGs. All points on the graph represent gene annotations. The DEGs are highlighted in blue and red based on the adjusted p-value of 0.05. Blue shows down-regulated DEGs, while red shows up-regulated DEGs. The mean difference plot contrasts parental GBC cells and highly metastatic GBC cells. The extreme values on the log fold change represent DEGs of higher expression levels. A greater fold change is observed in genes of lower mean expression profiles, while a lower fold change is observed in genes of a higher expression profile. The graph resembles the fanning effect because the DEGs move from right to left.

Heatmap: The heatmap (Fig. 4) reveals the expression levels of differentially expressed genes. Green and light green colours represent genes of lower expression levels, while red color represents genes of higher expression levels. The points of intersections represent the genes annotated on the x and y axes, while the third intersections generate the intensity of colours based on the gene matrix. The black colour represents no differentially expressed genes.

PPI network analysis: The PPI network analysis revealed ten hub genes (JUN, PTEN, VEGFA, TP53, AKT1, MYC, BCL2L1, CDKN1A, MMP9, and EGFR) that were essential in the progression of liver and gallbladder neoplasms. It was established that JUN regulates gene expression in response to growth factors, cytokines, and stress signals. PTEN is a tumor suppressor gene that encodes the PTEN protein, inhibiting cell growth and proliferation by regulating the PI3K/AKT signalling pathway. VEGFA encodes the VEGFA protein, promoting blood vessel growth and proliferation. TP53 encodes the p53 protein, a tumor suppressor that regulates the cell cycle and promotes cell death in response to DNA damage. AKT1 encodes the AKT1 protein, a kinase that plays a role in cell survival, proliferation, and metabolism. MYC encodes the c-Myc protein, a transcription factor regulating cell growth and proliferation. BCL2L1 encodes the Bcl-xL protein, which regulates cell survival. CDKN1A encodes the p21 protein, a cyclin-dependent kinase inhibitor promoting cell cycle arrest and tumor suppression. MMP9 encodes the matrix metalloproteinase nine protein, an enzyme that promotes tumor invasion and metastasis by degrading the extracellular matrix. EGFR encodes the epidermal growth factor receptor protein, a receptor tyrosine kinase regulating cell growth and proliferation (Fig. 5).

KEGG pathways: Present study established that the ten hub genes were significantly ($P<0.01$) enriched in various KEGG pathways such as C-type lectin receptor signalling (CLR), VEGF signalling, P53 signalling, NOD-like receptor signalling, apoptosis, necroptosis, cytokine-cytokine receptor interaction, salmonella infection, Herpes simplex virus I infection and MAPK signalling pathways. The CLR signalling pathway was associated with increased cytokine expression levels that increase T cell polarisation. Moreover, certain CLRs were also associated with activating the NF-kB and Toll-like receptors (Fig. 6).

P53 signalling pathway included transcription factors that regulated target genes involved in the progression of cell cycles, apoptosis, cell metabolism and repair of DNA tissues. P53 is responsible for tumor suppression in the liver and gallbladder.

The NOD-like receptor signalling pathway involved cytoplasmic recognition receptors (CRRs) that identify "non-self-components" such as microorganisms and DAMPs. This pathway is responsible for controlling the body's immune response.

Biological enrichment pathways: According to Fig. 7, the DEGs were enriched in biological pathways such as catabolism and metabolism of collagen, regulation of inflammatory response, cell death, defense response, apoptotic processes and programmed cell death.

Disease enrichment in different animal models: Magnitude of the p-values and Bonferroni corrections were considered to determine which animal has relatively better results. Smaller values suggest stronger evidence for the association. Comparing the p-values and Bonferroni corrections among the animals, it appears that Bonobo has the lowest values among the animals. These values indicate a very strong statistical significance ($P<0.0001$), suggesting a robust association between the hub genes and liver cancer in Bonobos. Therefore, Bonobo may have relatively better results based on these statistical measures (Table 1), followed closely by Mouse, Dog, Pig, and Rat.

DISCUSSION

It can be inferred from present analyses that QDD in treating liver and gallbladder neoplasms is associated with five hub genes (TP53, AKT1, PTEN, MMP-9 and VEGFA) significantly regulating the action of QDD. Furthermore, it was observed that the biological pathways of these genes were enriched in the catabolism and metabolism of collagen, regulation of inflammatory response, cell death, defense response, programmed cell death. The KEGG pathways were enriched in the C-type lectin receptor signalling pathway, VEGF signalling pathway, P53 signalling pathway, apoptosis and NOD-like receptor signalling pathway.

Table 1: Features of Dataset used in the study.

Dataset	Organism	Use	Parental gallbladder cancer cells	Metastatic gallbladder cancer cells	Type	Platform
GSE106671	Homo Sapiens	Integration Analysis	3	3	Expression profiling by array	GPL16956

Table 2: Disease enrichment in different animal models.

Animal model	Annotated Genes	Ref Genes	p value	Bonferroni Correction	Odds Ratio
Mouse	10	1017	2.77E-13	4.32E-10	Infinity
Dog	10	935	1.58E-13	2.45E-10	Infinity
Pig	10	919	1.62E-13	2.52E-10	Infinity
Bonobo	10	895	9.22E-14	1.43E-10	Infinity
Rat	10	955	1.62E-13	2.53E-10	Infinity

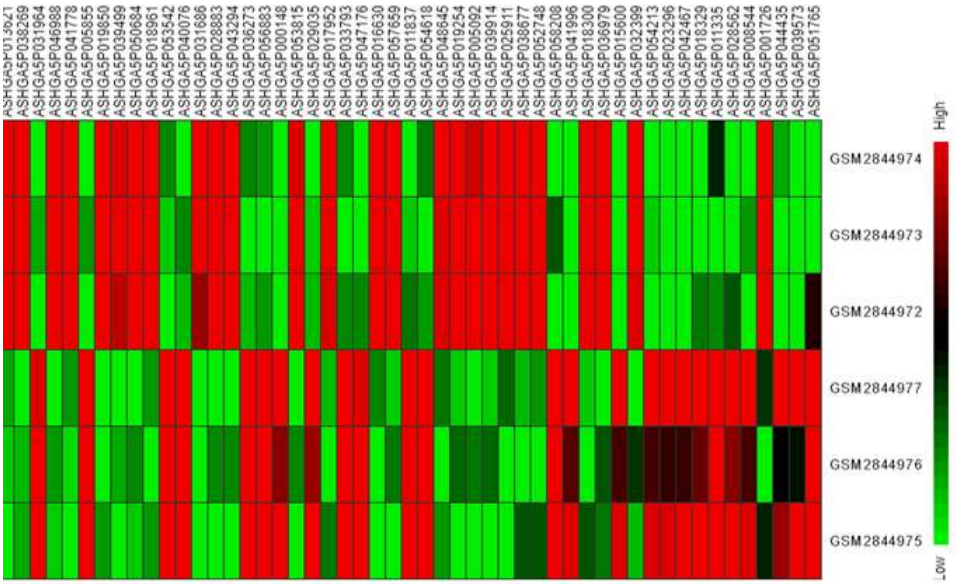

Fig. 4: Heatmap of Differentially Expressed Genes

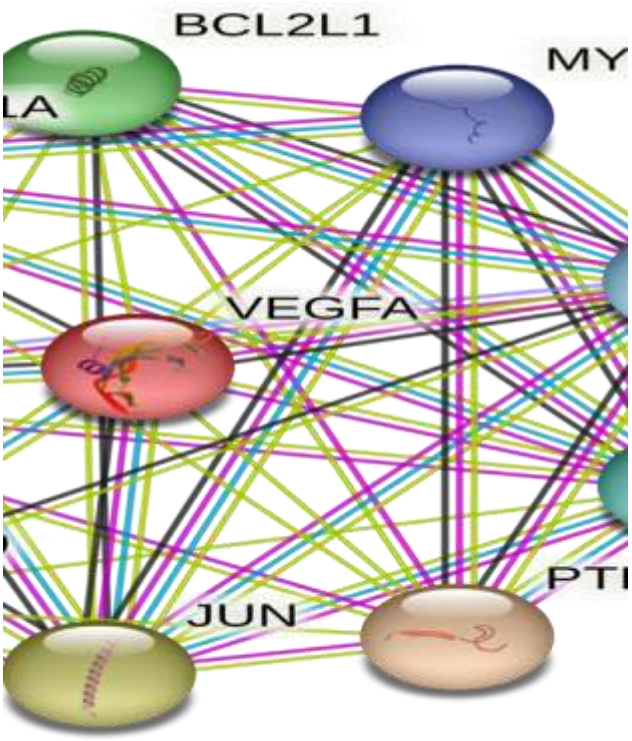

Fig. 5: PPI Networks showing hub genes associated with QDD in liver and gallbladder neoplasms.

The catabolism and metabolism of collagen pathways had the greatest fold enrichment. Collagen fibers are produced by myofibroblasts and offer a protective cover to the liver and gallbladder (Mederacke *et al.*, 2013; Puche *et al.*, 2013). Myofibroblasts are mainly produced by hepatic stem cells; however, the migration and change in polarity of epithelial stem cells could lead to the production of myofibroblasts through epithelial-mesenchymal transition (EMT) (Puche *et al.*, 2013). Fibrocytes consist of spindles and express fibroblast markers such as collagen type I, vimentin and fibronectin (de Oliveira and Wilson 2020; Ozono *et al.*, 2021). Moreover, fibrocytes also increase the expression of hematopoietic cell markers such as CCR5, CD80, CD86 and CD11b (de Oliveira and Wilson 2020; Ozono *et al.*, 2021; Shimoda *et al.*, 2021).

Collagen is a significant component of the extracellular matrix (ECM), offering structural support to the liver and gallbladder. The pathways of catabolism and collagen metabolism involve collagen breakdown within the ECM. Dysregulation of these pathways enhances the growth and development of gallbladder and liver neoplasms. For example, the metastasis and invasion of tumors, angiogenesis and growth of tumors, and tumor microenvironments. In metastasis and invasion of tumors, collagen fibers act as a physical barrier to the tumor cells; therefore, collagen breakdown in the presence of enzymes such as matrix metalloproteinases (MMPs) permits the tumors to enter the surrounding cells, tissues and the lymphatic system. Therefore, increased levels of MMPs enhance the growth and progression of gallbladder and liver neoplasms. It was observed that inhibiting the activity of MMP lowers and eliminates tumor invasion and metastasis.

Present study conducted limited research on how QDD alters the catabolism and metabolism of collagen pathways in liver and gallbladder neoplasms. However, studies by Wang *et al.* (2017), Tao *et al.* (2021) and Kitano *et al.* (2016) have suggested that *Salvia miltiorrhiza*,

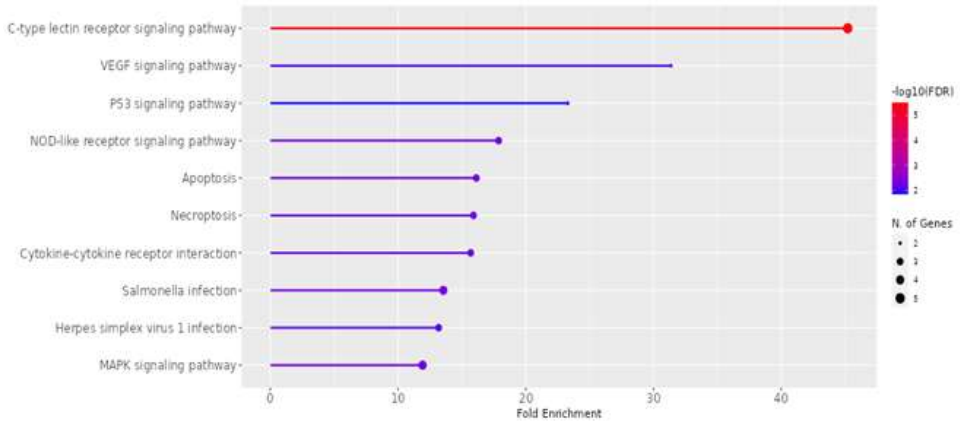

Fig. 6: KEGG Pathways of the hub genes

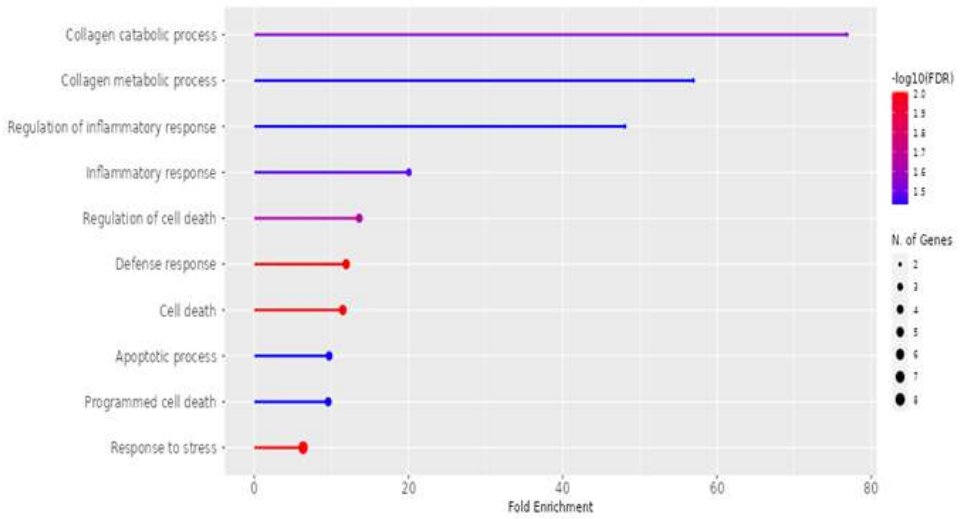

Fig. 7: Biological pathways

which is added in QDD, inhibits the expression levels of collagen I and III in hepatic stellate cells resulting in inhibition of the development of liver fibrosis and subsequent liver cancer. Also, it reduces liver fibrosis in animal models of liver injury and inhibits cell proliferation in liver cancer. Similarly, studies by Ma *et al.* (2015), Lin *et al.* (2015) and Lee *et al.* (2009) suggested that *Gardenia jasminoides* inhibits the expression of collagen I and III in skin fibroblasts through anti-inflammatory and antioxidant effects.

It is observed in present study that catabolism and collagen metabolism is enhanced during angiogenesis and growth of tumors. Collagen fibers act as a scaffold for the growth and development of tissues and blood vessels. Hence, the breakdown of collagen through enzymes such as lysyl oxidase (LOX) increases the growth of tumors. Therefore, it is observed that limiting the activity of LOX reduces the growth and progression of gallbladder and liver neoplasms.

It can be inferred from present analyses that catabolism and collagen metabolism regulate the microenvironment of gallbladder and liver neoplasms. This is because collagen fibres are involved in numerous interactions with immune cells, pro-inflammatory cytokines and various components of ECM associated with controlling immune response and promoting the survival of tumors (Mhaidly and Mechta-Grigoriou, 2021). Therefore, it can be suggested that a dysregulation of collagen metabolism would eliminate these interactions and increase immunosuppressive components in the liver and gallbladder tumor microenvironments.

Our study established that regulation of inflammatory response was a significant biological pathway in liver and gallbladder neoplasms. Regulation of inflammatory responses refers to a complicated biological pathway involving signalling pathways, cytokines and immune cells (Chen *et al.*, 2018). For example, chronic inflammations, signalling of cytokines and immunosuppression. Chronic inflammation significantly enhances liver and gallbladder neoplasms in conditions like alcohol, viral hepatitis and non-alcoholic steatohepatitis (NASH). Chronic inflammation leads to the activation of immune cells by recruiting and activating macrophages, neutrophils and T cells that produce chemokines and cytokines that increase tissue damage. Moreover, the DNA is also damaged, and mutations are introduced due to the production of reactive oxygen species. The presence of inflammatory cytokines activates oncogenesis and deactivates the tumor-suppressing genes.

It is observed that chronic inflammation enhances fibrosis, where excess collagen is deposited in the liver tissues. Fibrosis creates a stiff environment which increases tumor growth and inhibits immune cell infiltration into the microenvironment (Winkler *et al.*, 2020). The production of inflammatory cytokines such as TNF-alpha and IL-6 increases the production of angiogenic factors such as vascular endothelial growth factors (VEGF), which increases the growth and development of liver and gallbladder neoplasms.

There was limited research on the specific action of QDD on liver and gallbladder neoplasms. Lee *et al.* (2009)

suggested that *Gardenia jasminoides* have anti-inflammatory effects that inhibit pro-inflammatory cytokines such as TNF-α and IL-1β in acute pancreatitis and colitis. Moreover, *Scutellaria baicalensis* limits the activity of pro-inflammatory cytokines (for example, TNF-α, IL-6) in mouse models of rheumatoid arthritis.

Our study suggests that dysregulation of inflammatory response is critical in reducing the growth and progression of neoplasms. In chronic hepatitis B and C, the persistent inflammatory response can lead to liver cirrhosis and an increased risk of hepatocellular carcinoma (HCC) (D'souza *et al.*, 2020). Also, in NASH, the inflammatory response in the liver can lead to the development of non-alcoholic fatty liver disease (NAFLD), which increases the risk of HCC. Moreover, we established that targeting the regulation of the inflammatory response pathway, such as using immune checkpoint inhibitors or inhibiting cytokine signalling, can lead to the novel treatment of liver and gallbladder neoplasms.

Immunosuppression increases the growth and development of liver and gallbladder neoplasms because tumor cells can escape the immune system by increasing the expression levels of immune checkpoint molecules, such as PD-L1, inhibiting activation and proliferation of T cells. T cells are critical in the recognition and removal of tumor cells, and inhibiting immune checkpoints increases the growth and development of neoplasms (Chiu *et al.*, 2020). Furthermore, immunosuppression increases the recruitment of suppressive cells, for example, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) that are released into the tumor microenvironment (Haist *et al.*, 2021). Additionally, it was observed that inhibiting the functions of dendritic cells plays a key role in enhancing the tumor antigens to T cells and developing an anti-tumor response. However, the inflammatory cytokines can limit the activity of dendritic cells and impair the presentation of antigens and activation of T cells.

Immunosuppression of tumor microenvironments increases the progression of liver and gallbladder neoplasms by inhibiting the proliferation and activation of T cells and the functions of dendritic cells. We suggest that by targeting the immune suppression components, a suitable strategy can be achieved in diagnosing and treating liver and gallbladder neoplasms. We observe that immune checkpoint inhibitors and therapies targeting the MDSCs and Tregs are potential biomarkers for treating liver and gallbladder neoplasms.

Present study confirmed that regulating cell death biological pathways was critical in developing and progressing liver and gallbladder neoplasms. The cell death pathway involves autophagy, necroptosis and apoptosis. Apoptosis is a form of programmed cell death associated with eliminating damaged and mutated cells (Wu *et al.*, 2020). The dysregulation of apoptosis increases the build-up of damaged cells associated with liver and gallbladder neoplasms. For instance, mutations of tumor suppressor TP53 impair apoptosis. Also, necroptosis is a programmed cell death initiated by viral infections and inflammations. The dysregulation of necroptosis enhances chronic inflammation and tissue damage. We also found that dysregulation of autophagy increases the accumulation of mutated and damaged cells, increasing the progression of neoplasms. We suggest that dysregulation of the regulation of the cell death pathway can provide new insights into the development and progression of liver and gallbladder neoplasms leading to new therapies.

We observed limited research on the action of QDD in regulating the cell death pathway in the gallbladder and liver neoplasms. However, our findings were consistent with Bie *et al.* (2017), Li *et al.* (2018) and Hu *et al.* (2016), who suggested that *Scutellaria baicalensis* is capable of inducing apoptosis and inhibiting cell infiltration and proliferation; a similar effect was observed with *Gardenia jasminoides*. Miraj *et al.* (2016) observed that *Astragalus membranaceus*, a critical component of QDD, induces apoptosis and enhances the sensitivity of cancer cells to chemotherapeutic drugs.

Our study established that the DEGs were enriched in KEGG pathways of C-type lectin receptor (CLR) signalling, VEGF signalling pathway, TP53 signalling pathway and NOD-like receptor signalling pathway. The CLR signalling pathway is a complicated pathway associated with the response and recognition of foreign substances in the immune cells. The pathway utilizes several mechanisms, such as tumor-associated macrophages (TAMs), dendritic cells and immune evasion of tumors. TAMs consist of infiltrating immune cells within the tumor microenvironment that increases the progression of neoplasms. TAMs are produced by circulating monocytes or tissue-resident macrophages. The CLRs consist of pattern recognition receptors (PRRs) expressed on the surface of immune cells. When CLRs on macrophages recognize specific ligands, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), they initiate a downstream signalling cascade that activates macrophages and secretes pro-inflammatory cytokines and growth factors.

VEGF, which increases the production of blood vessels within the tumor microenvironment, permits the tumor cells to obtain oxygen and nutrients. Furthermore, TAMs can produce MMPs responsible for extracellular matrix degradation and promoting neoplasms. Our findings were consistent with Hara and Saito (2009), who investigated the role of the CLR pathway in activating and recruiting TAMs in HCC models of a mouse. They established that inhibiting the CLR pathway limits the progression of liver and gallbladder neoplasms. Similarly, Hong *et al.* (2018) suggested that TAMs have a crucial role in the progression and metastasis of ICC. They reported that TAMs were overexpressed in the CLR pathway of Dectin-1; hence, Dectin-1 can be targeted using unique antibodies to lower the activation and recruitment of TAMs, which inhibits metastasis. According to Cao (2013), the expression of cytokine VEGF is critical in promoting angiogenesis and inhibiting the action of VEGF using specific antibodies, resulting in the growth of neoplasms.

We observed limited research on the action of QDD on the CLR signalling pathway. However, other studies by Dong *et al.* (2016), Xiang *et al.* (2022) and Sung *et al.* (2014) have found that *Scutellaria baicalensis*, which is included in QDD, inhibits the activation of the CLR pathway in a mouse model of acute lung injury by reducing the production of pro-inflammatory cytokines and chemokines. Similarly, the herb *Gardenia jasminoides*

reduces the expression of the CLR pathway receptor Dectin-1 in a mouse model of acute pancreatitis, reducing inflammation and tissue damage. We propose that modulating the CLR pathway by constituents of QDD enhances its anti-inflammatory and anti-tumor effects in liver and gallbladder neoplasms.

The PPI networks revealed ten hub genes (JUN, PTEN, VEGFA, TP53, AKT1, MYC, BCL2L1, CDKN1A, MMP9, and EGFR) that were significant in the progression of liver and gallbladder neoplasms. We established that the JUN gene was responsible for encoding the c-Jun proteins that constitute the transcription factor Activator Protein 1 (TFAP1) that controls the expression levels of genes in response to stress signals, inflammatory cytokines and growth factors. Miao *et al.* (2022) observed that the JUN/JNK signalling pathway could be blocked by protein kinase inhibitor TT-00420. Similarly, Liu *et al.* (2017) showed that c-Jun is critical in developing liver and gallbladder neoplasms. Our findings were similar to Zhang *et al.* (2016) and Jin *et al.* (2015), who suggested that the expression of JUN was increased in HCC tissues with a knockdown effect of inhibiting the progression of HCC in liver cells. Furthermore, they established that c-Jun has an essential role in CCA because its overexpression limits the growth and development of tumors. Therefore, we propose that c-Jun could be exploited as a significant biomarker and therapeutic target in treating liver and gallbladder neoplasms.

We also found that phosphatase and tensin homolog (PTEN) gene was associated with encoding tumor suppression proteins (p53, retinoblastoma, NF1 and BRCA1 & 2) that control the P13K/AKT signalling of cellular growth and metabolism. Xu *et al.* (2006) observed that PTEN is essential in the growth and progression of gallbladder and liver neoplasms. Moreover, Squarize *et al.* (2013) observed that a down-regulation of PTEN increases tumor aggression leading to poor prognosis. Also, it inhibited the activity of HCC cellular growth and migration. Roa *et al.* (2016) reported that PTEN plays a critical role in developing GBC in the gallbladder. They suggested that the expression levels of PTEN were significantly reduced in GBC tissues compared to normal tissues.

We observed limited research on the action of QDD and the expression levels of all hub genes. However, certain herbs used in the composition of QDD have significant effects on these hub genes and their corresponding pathways. For instance, *Scutellaria baicalensis* limits the activity of AKT1 and downstream targets in liver cells. Similarly, *Astragalus membranaceus* increases the upregulation and expression of PTEN and TP53. In contrast, *Astragalus membranaceus* increases the downregulation and expression of MYC in liver cells. Our study suggests that *Salvia miltiorrhiza* downregulates VEGFA in liver cells and reduces angiogenesis. Additionally, *Gardenia jasminoides* was linked with limiting the activation of EGFR-corresponding signalling pathways.

Conclusions: It can be concluded from present investigations that the network pharmacology of QDD is enriched in various biological pathways, such as the catabolism and metabolism of collagen, regulation of inflammatory response, cell death, defence response, apoptotic processes and programmed cell death. Moreover, the DEGs expressed in gallbladder and liver neoplasms were enriched in KEGG pathways of C-type lectin receptor (CLR) signalling, VEGF signalling pathway, TP53 signalling pathway and NOD-like receptor signalling pathway. QDD exerts anti-tumor effects by targeting these pathways through its component herbs, such as *Scutellaria baicalensis* or *Astragalus membranaceus.* We established that the composition of QDD varies depending on various formulations.

The bioinformatics of QDD in liver and gallbladder neoplasms identified ten hub genes associated with neoplasm progression. However, these hub genes were regulated by various herbs used to formulate QDD. Currently, there is limited research on the specific mechanisms by which various herbs increase or decrease the expression levels of these hub genes in gallbladder and liver neoplasms.

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