Original Article

Transcriptomic and network pharmacology approaches revealed possible mechanisms underlying the 5-fluorouracil (5-FU)-sensitizing effect of Xuan-Fu-Hua decoction treatment on liver cancer cells

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Background: Xuan-Fu-Hua decoction is a traditional Chinese medicine formula widely used for the treatment of inflammation-related disease in the lung and liver. This study aimed to investigate the effect of Xuan-Fu-Hua decoction treatment on liver cancer cells and its mechanism of action.

Methods: The impact of Xuan-Fu-Hua decoction treatment on the proliferation and apoptosis of SMMC-7721 liver cancer cells with or without 5-fluorouracil (5-FU) cotreatment was determined in both *in vitro* and *in vivo* settings. Alterations in gene expression patterns in SMMC-7721 cells induced by Xuan-Fu-Hua decoction treatment were explored by transcriptomic sequencing. Effective components of Xuan-Fu-Hua decoction and their target proteins were investigated using network pharmacology approaches.

Results: Xuan-Fu-Hua decoction alone did not significantly influence SMMC-7721 liver cancer cell growth, but it significantly increased the 5-Fu-induced growth inhibition and apoptosis of SMMC-7721 liver cancer cells *in vitro* and *in vivo*. Most differentially expressed genes in SMMC-7721 liver cancer cells with or without Xuan-Fu-Hua decoction treatment were enriched in cell apoptosis-related pathways. Xuan-Fu-Hua decoction treatment significantly increased the transcription levels of *DDIT3*, *PMAIP1*, and *ZMAT3* genes while decreasing that of *WNT4*, *AXIN2*, *NFE2L2*, *TGFBR1*, *MITF*, and *IGFBP3* genes. An interaction network between the effective components and their possible target proteins was constructed by predicting compound-target protein and protein-protein interactions. Gene set enrichment analysis revealed the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway as well as Bcl-2 and Mcl-1 proteins as potential regulatory targets of Xuan-Fu-Hua decoction in sensitizing SMMC-7721 cells to the cytotoxicity of 5-FU treatment.

Conclusions: Xuan-Fu-Hua decoction increased the sensitivity of liver cancer cells to the cytotoxicity of 5-FU treatment, possibly by potentiating cell apoptosis and inhibiting the prosurvival machinery.

Keywords: Liver cancer; Xuan-Fu-Hua decoction; transcriptomic sequencing; network pharmacology

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Introduction

The development of chemotherapeutic resistance is a major obstacle in liver cancer management. Liver cancer cells have been reported to develop various mechanisms to evade apoptosis and other forms of cell death induced by chemotherapeutic drugs, such as the hyperactivation of WNT/beta-catenin signaling, transforming growth factor (TGF)-beta signaling, and hepatocyte growth factor (HGF)-mesenchymal-epithelial transition (MET) signaling. Activation of these signaling pathways increases the transcription of antiapoptotic or prosurvival genes while repressing that of genes responsible for the execution or positive regulation of cell apoptosis (1-4). The inhibition of signal transduction mechanisms has thus been considered a possible approach to sensitizing liver cancer cells to chemotherapy and improving patient prognosis.

Xuan-Fu-Hua decoction is a traditional Chinese medicine formula commonly used for the treatment of "stasis of the liver (Gan Zhuo)"-related diseases, such as liver fibrosis, cholecystitis, and fatty liver (5,6). Xuan-Fu-Hua decoction is mainly composed of 3 herbs, namely Xuan-Fu-Hua (Inulae Flos), Qian-Cao (*Rubia cordifolia*), and Cong-Bai (Allii Fistulost Bulbus), of which Xuan-Fu-Hua and Qian-Cao are considered to be the main medicinal components of this formula. Some traditional Chinese medicine practitioners consider the development of liver cancer in line with the symptoms of "stasis of the liver", and the treatment of liver cancer with Xuan-Fu-Hua decoction has been found effective in clinical practice. Xuan-Fu-Hua decoction also has been co-applied with chemotherapy drugs and shown certain advantages in clinical practice. However, the mechanism of Xuan-Fu-Hua decoction treatment in liver cancer management remains underexplored. Network pharmacology is a novel approach for investigating the therapeutic mechanism of traditional Chinese medicine (7-9). In this study, we evaluated the effect of Xuan-Fu-Hua decoction treatment on liver cancer cells *in vitro* and *in vivo*, and we combined the transcriptomic sequencing and network pharmacology techniques to reveal the mechanism of action of Xuan-Fu-Hua decoction treatment in liver cancer cells. Our results suggested that Xuan-Fu-Hua decoction treatment increased the sensitivity of liver cancer cells to 5-fluorouracil (5-FU) treatment by potentiating the cell apoptosis pathway, possibly by increasing the expression of proapoptotic genes, reducing prosurvival gene expression, and inhibiting phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-1814/rc).

Methods

Preparation of Xuan-Fu-Hua decoction

The same mass of Xuan-Fu-Hua and Qian-Cao were mixed and extracted twice by boiling and refluxing with 5 times the mass of distilled water for 30 minutes each time. The 2 extracts were combined and filtered to remove insoluble impurities, after which the filtrate was freeze-dried and stored at –20 ℃. The freeze-dried powder was redissolved at room temperature in phosphate-buffered saline (PBS) at 5 mg/mL as a stock solution prior to use at 50 μg/mL for *in vitro* assays and 500 mg/kg bodyweight for *in vivo* experiments (according to the pharmacological experimental scheme, the daily dose of Xuan-Fu-Hua decoction was 30 g crude drug for adults with a body weight of 60 kg).

Cell culture and treatment

SMMC-7721 liver cancer cells were purchased from Procell (CL-0216, Wuhan, China). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (PM150110, Procell) supplemented with 10% fetal bovine serum (164210-500, Procell) and 1% penicillin-streptomycin stock solution (PB180120, Procell). Cell culture and treatment were performed in a humidified cell incubator at 37 ℃ with 5% CO_2 atmosphere. The cells were subcultured every 3 days and were assayed at about 70% confluency within 5 passages, unless otherwise indicated.

Cell growth and apoptosis assay in vitro

Overall cell growth of SMMC-7721 cells was evaluated using Cell Counting Kit-8 (CK04, Dojindo, Shanghai, China). The cells in logarithmic growth phase were inoculated at a density of 5,000 cells per well in 96-well

plates. After 2 hours, except for the control group cells (Ctrl group), the cells were incubated with Xuan-Fu-Hua decoction (XFH group), 5-FU (5-FU group), or Xuan-Fu-Hua decoction plus 5-FU (5-FU + XFH group) for 24, 48, and 72 hours, after which the viable cells in each well were compared by incubation with WST-8 working solution for 1 hour and subsequent colorimetric analysis using a microplate reader. Apoptosis of SMMC-7721 cells was evaluated using Annexin V, FITC Apoptosis Detection Kit (AD10, Dojindo). The cells in logarithmic growth phase cultured in 6-well-plates were treated with Xuan-Fu-Hua decoction, 5-FU, or Xuan-Fu-Hua decoction plus 5-FU for 24 hours, after which the cells were subject to Annexin V-FITC/propidium iodide (PI) double staining, following the manufacturer's instructions, and flow cytometry. The cells with Annexin V-FITC + PI staining were considered apoptotic cells. The *in vitro* assays were performed in 3 biological replicates with 2 technical replicates in each.

Xenograft tumor model establishment and assays

Balb/c female nude mice aged 4 weeks (22.14±3.28 g) were purchased from Cyagen Biosciences (Guangzhou, China) and were kept under sterile conditions. A xenograft tumor model was established by subcutaneous injection of 5×10^6 SMMC-7,721 cells resuspended in 200 µL of PBS. The mice were divided randomly into 4 groups (n=4) when tumors reached 80–120 mm^3. Every other day, the tumor-bearing mice received either intraperitoneal injection of normal saline (Ctrl group), Xuan-Fu-Hua decoction gavage (XFH group), intraperitoneal injection of 5-FU gavage (5-FU group), or Xuan-Fu-Hua decoction gavage plus 5-FU treatment (5-FU + XFH group), during which time the body weight and tumor volume were monitored. The tumor-bearing mice were euthanized 6 weeks after treatment, and the xenograft tumors were collected for weighing. Intratumoral cell apoptosis was further evaluated by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling using *in situ* cell death detection kit (11684795910, Sigma-Aldrich, Shanghai, China) following the manufacturer's instructions. The *in vivo* assays were performed in 2 biological replicates with 2 technical replicates in each. The animal experiment was approved by the ethics review committee of Henan University of Chinese Medicine (No. DWLLGZR20200412), in compliance with Henan University of Chinese Medicine guideline for the care and use of animals. A protocol was prepared without registration before the study.

Transcriptomic sequencing and data analysis

Transcriptomic sequencing of SMMC-7721 cells with or without Xuan-Fu-Hua decoction treatment and the preprocessing of sequencing data were performed by LC-Bio Technologies (Hangzhou, China). Genes in SMMC-7721 cells with more than two-fold differences in their transcription levels after Xuan-Fu-Hua decoction treatment compared to nontreated cells with P values less than 0.05 were considered significantly differentially expressed genes and were subject to gene set enrichment analysis performed using the search tool for the retrieval of interacting genes/proteins (STRING) online platform (v11).

Prediction of possible target proteins of Xuan-Fu-Hua decoction effective components

All components of Xuan-Fu-Hua and Qian-Cao documented in the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP) were downloaded, and those with oral bioactivity >30% and drug-like index >0.18 were considered effective components. The putative targets of these effective components were predicted using the SwissTargetPrediction platform, with interaction possibility higher than 0.5. After deduplication, an interaction network comprising all effective components of the 2 herbs and their corresponding target proteins was constructed using Cytoscape software (v3.8.2). Gene set enrichment analysis of genes corresponding to potential target proteins of the Xuan-Fu-Hua decoction effective components was performed using the STRING online platform (v11).

Statistical analysis

Statistical analysis of the experimental results was performed using GraphPad Prism software (v9.0). One-way or two-way analysis of variance with Tukey's test as the post-hoc test was used for multigroup comparison. A difference was considered statistically significant when $P<0.05$.

Results

Xuan-Fu-Hua decoction cotreatment increased the sensitivity of SMMC-7721 liver cancer cells to 5-FU-induced apoptosis

We first evaluated the influence of Xuan-Fu-Hua decoction intervention on liver cancer cells. Our preliminary

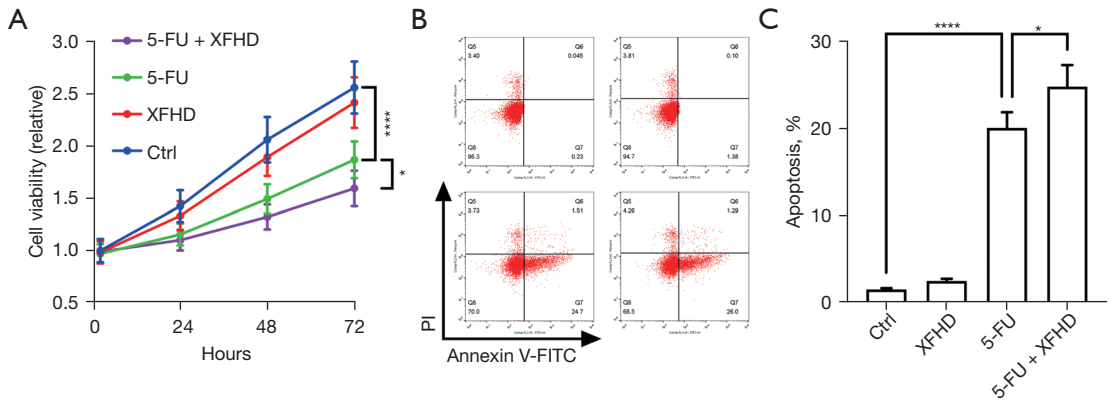

Figure 1 Xuan-Fu-Hua decoction cotreatment sensitized SMMC-7721 liver cancer cells to 5-FU treatment *in vitro*. (A) SMMC-7721 cells were treated with PBS (Ctrl), Xuan-Fu-Hua decoction, 5-FU, or 5-FU plus Xuan-Fu-Hua decoction for 24, 48, or 72 hours, after which the viability of cells were compared by CCK-8 assay. All data were normalized to the mean value of the Ctrl group at the initial timepoint. (B,C) SMMC-7,721 cells were treated with PBS (Ctrl), Xuan-Fu-Hua decoction, 5-FU, or 5-FU plus Xuan-Fu-Hua decoction for 24 hours, after which the cells were subject to fluorescent staining of Annexin V/PI and flow cytometry. Cells with Annexin V^+/PI^- staining were considered apoptotic cells. Data in A and C are presented as mean ± SD. *, P<0.05, ****, P<0.0001. 5-FU, 5-fluorouracil; XFHD, Xuan-Fu-Hua decoction; Ctrl, control; V-FITC, Annexin V-fluorescein isothiocyanate; PBS, phosphate-buffered saline; CCK-8, Cell Counting Kit-8; PI, prodium iodide; SD, standard deviation.

experimental results showed that the impact of Xuan-Fu-Hua decoction treatment on overall cell growth or apoptosis of SMMC-7721 liver cancer cells *in vitro* was minimal. Interestingly, however, cotreatment with Xuan-Fu-Hua decoction significantly augmented the 5-FU challenge-induced growth inhibition and apoptosis in SMMC-7721 liver cancer cells *in vitro* compared to the effect of treatment with 5-FU alone (*Figure 1*). We then constructed a xenograft tumor model to verify these results *in vivo*. We found that oral administration of Xuan-Fu-Hua decoction alone did not significantly affect xenograft tumor growth and intratumoral cell apoptosis, but it significantly enhanced the growth-inhibiting and apoptosis-inducing effect of 5-FU on the xenograft tumor in the cotreatment setting (*Figure 2*). These results suggested that Xuan-Fu-Hua decoction could increase the sensitivity of liver cancer cells to chemotherapy.

Impact of Xuan-Fu-Hua decoction treatment on the transcriptome of SMMC-7721 liver cancer cells was revealed by next-generation sequencing and gene set enrichment analysis

To investigate how Xuan-Fu-Hua decoction treatment affected liver cancer cells, we compared the transcriptome of SMMC-7721 cells before and after Xuan-Fu-Hua decoction treatment to identify genes with expression levels significantly affected by Xuan-Fu-Hua decoction treatment (available at https://cdn.amegroups.cn/static/public/tcr-22-1814-1.xlsx). We found that in SMMC-7721 cells induced by Xuan-Fu-Hua decoction treatment, the transcription levels of 425 genes were significantly increased and that of 232 genes were significantly decreased (*Figure 3A*). The 425 significantly upregulated genes included 347 protein-coding genes and 78 noncoding genes, and in the 232 significantly downregulated genes, there were 203 coding genes and 29 noncoding genes. Enrichment analysis of the protein-coding genes combined was performed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases to further explore the specific impact of Xuan-Fu-Hua decoction treatment on SMMC-7721 cells. After filtering the GO and KEGG terms enriched by the 550 protein-coding genes using a false discovery rate <0.05 threshold, we found that these genes were significantly enriched in only 2 KEGG terms, namely the "pertussis" and "complement and coagulation cascades" pathways. The GO term enrichment analysis results showed that these 550 protein-coding genes were most significantly enriched in biological processes associated with the execution and regulation of cell apoptosis (*Figure 3B-3D*). Notably, we

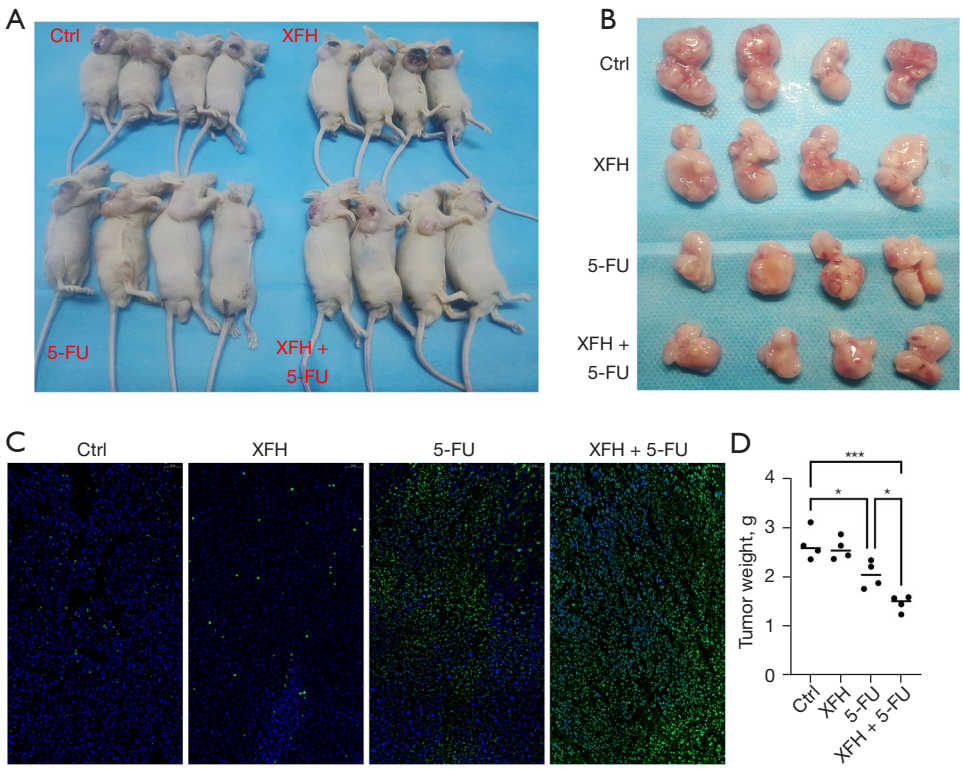

Figure 2 Xuan-Fu-Hua decoction cotreatment enhanced the antitumor effect of 5-FU treatment in vivo. The tumor-bearing Balb/c mice were either left untreated or treated with indicated interventions every 2 days for 6 weeks, after which the mice were euthanized (A), and the xenograft tumors were collected for weighing (B,D) and TdT-mediated dUTP nick-end labeling evaluated cell apoptosis *in situ*, magnification: 20× (C). The mean value of each group of data in D is marked by a short line. *, P<0.05; ***, P<0.001. 5-FU, 5-fluorouracil; XFH, Xuan-Fu-Hua; Ctrl, control; TdT, terminal deoxyribonucleotidyl transferase; dUTP, deoxy-uridine triphosphate.

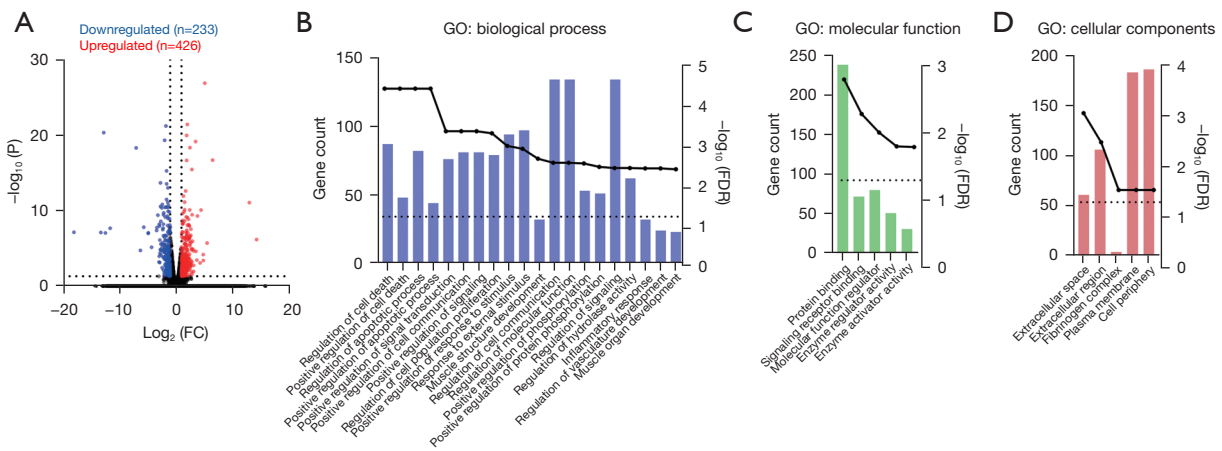

Figure 3 Transcriptomic sequencing and gene set enrichment analysis results. (A) 426 significantly upregulated genes and 233 significantly downregulated genes in SMMC-7,721 cells caused by Xuan-Fu-Hua decoction treatment were identified. (B-D) GO enrichment results of the differentially expressed genes. The columns represent the number of genes from the differentially expressed genes that were mapped to each GO term, and the dotted line indicates the ranking of –log10 (FDR) value. The horizontal dashed line indicates P=0.05 threshold. FC, fold change; GO, Gene Ontology; FDR, false discovery rate.

found that Xuan-Fu-Hua decoction treatment significantly increased the transcription of *DDIT3*, *PMAIP1*, and *ZMAT3* genes, which encode proteins known to antagonize the antiapoptotic role of Bcl-2 and Bcl-xl proteins on the mitochondrial membrane and participate in the cytochrome c release from mitochondria into the cytosol. At the same time, the transcription levels of *WNT4*, *AXIN2*, *NFE2L2*, *TGFBR1*, *MITF*, and *IGFBP3* genes were significantly downregulated in SMMC-7721 cells by Xuan-Fu-Hua decoction treatment. These results suggested that Xuan-Fu-Hua decoction treatment might have increased the sensitivity of liver cancer cells to 5-FU challenge, possibly by potentiating the intrinsic apoptosis pathway.

Network pharmacology analysis of potential target proteins of Xuan-Fu-Hua decoction effective components

To investigate the molecular mechanism underlying the aforementioned effects of Xuan-Fu-Hua decoction treatment, we obtained all currently known components in Xuan-Fu-Hua and Qian-Cao documented in the TCMSP online database, and those with oral bioactivity >30% and drug-like index >0.18 were considered effective components. We found that Xuan-Fu-Hua and Qian-Cao each contained 19 possible effective components, with 1 active ingredient, MOL000358 (beta-sitosterol), being common to both (Table S1). We hypothesized that the effective components in Xuan-Fu-Hua decoction exerted their medicinal properties by interacting with intra- or intercellular biomolecules and influencing the biological functions of the latter. We employed the SwissTargetPrediction online platform to explore biomolecules potentially interacting with these Xuan-Fu-Hua decoction effective components with an interaction possibility higher than 50%. We found a total of 107 possible target proteins corresponding to 7 effective components in Xuan-Fu-Hua, and we found 10 possible target proteins associated with 5 effective components in Qian-Cao (available at https://cdn.amegroups.cn/static/public/tcr-22-1814-2.pdf). The 6 possible target proteins shared by the effective components in both herbal ingredients were CYP19A1, AR, HMGCR, CYP51A1, NPC1L1, and NR1H3. The interaction between the effective components from the 2 herbs and their target proteins is shown in *Figure 4A*.

We further envisaged that the effective components in Xuan-Fu-Hua and Qian-Cao may sensitize liver cancer cells to 5-FU challenge-induced apoptosis by interacting with the potential target proteins and influencing the activation of the signaling pathways with which the latter are associated. We therefore performed enrichment analysis of the 111 genes corresponding to the potential target proteins of the effective components in Xuan-Fu-Hua decoction ingredients using GO, KEGG, and Reactome pathway databases (*Figure 4B-4F*). Among the top 20 most significantly enriched GO biological process terms, we found that the potential target proteins of Xuan-Fu-Hua and Qian-Cao effective components may be involved in cellular response to oxygen-containing compound, intracellular signal transduction, as well as the execution and regulation of cell apoptosis. The GO cellular component term enrichment results showed that the majority of the potential target proteins of Xuan-Fu-Hua decoction effective components were located in the cytosol and plasma membrane. KEGG enrichment analysis results revealed that the genes corresponding to the potential target proteins of Xuan-Fu-Hua decoction effective components were most significantly enriched in signaling pathways that are known to drive liver cancer progression, including the PI3K/AKT signaling pathway, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor resistance pathway, and endocrine resistance pathway. These genes included *FLT3*, *KDR*, *CDK6*, *CDK2*, *IGF1R*, *EGFR*, *INSR*, *MET*, *GSK3B*, *PTK2*, *PKN1*, *PIK3CG*, *MCL1*, *SYK*, *BCL2*, *PIK3R1*, *AKT1*, *AXL*, *SRC*, *MMP2*, *ESR2*, and *MMP9*. Reactome pathway enrichment results further confirmed the significant involvement of the genes corresponding to the potential target proteins of Xuan-Fu-Hua decoction effective components in the PI3K/AKT signaling pathway. These genes included *CSNK2A1*, *EGFR*, *INSR*, *TNKS*, *MET*, *GSK3B*, *ESR2*, *TNKS2*, *SRC*, *PIK3R1*, and *AKT1*. We found that the majority of the proteins listed above were the target of effective component MOL000098 (Quercetin) in Xuan-Fu-Hua, while the effective component MOL006160 (Alizarin) from Qian-Cao may also interact with the Bcl-2 and Mcl-1 proteins involved in the PI3K/AKT signaling pathway. Interestingly, we also found that the effective component MOL000006 (Luteolin) in Xuan-Fu-Hua might interact with tankyrase and tankyrase 2 proteins corresponding to *TNKS* and *TNKS2* genes that were implicated in the PIP3-activated AKT signaling pathway in the Reactome pathway enrichment results.

Discussion

In Chinese medicine practice, Xuan-Fu-Hua decoction is mainly used to treat inflammation-associated liver and

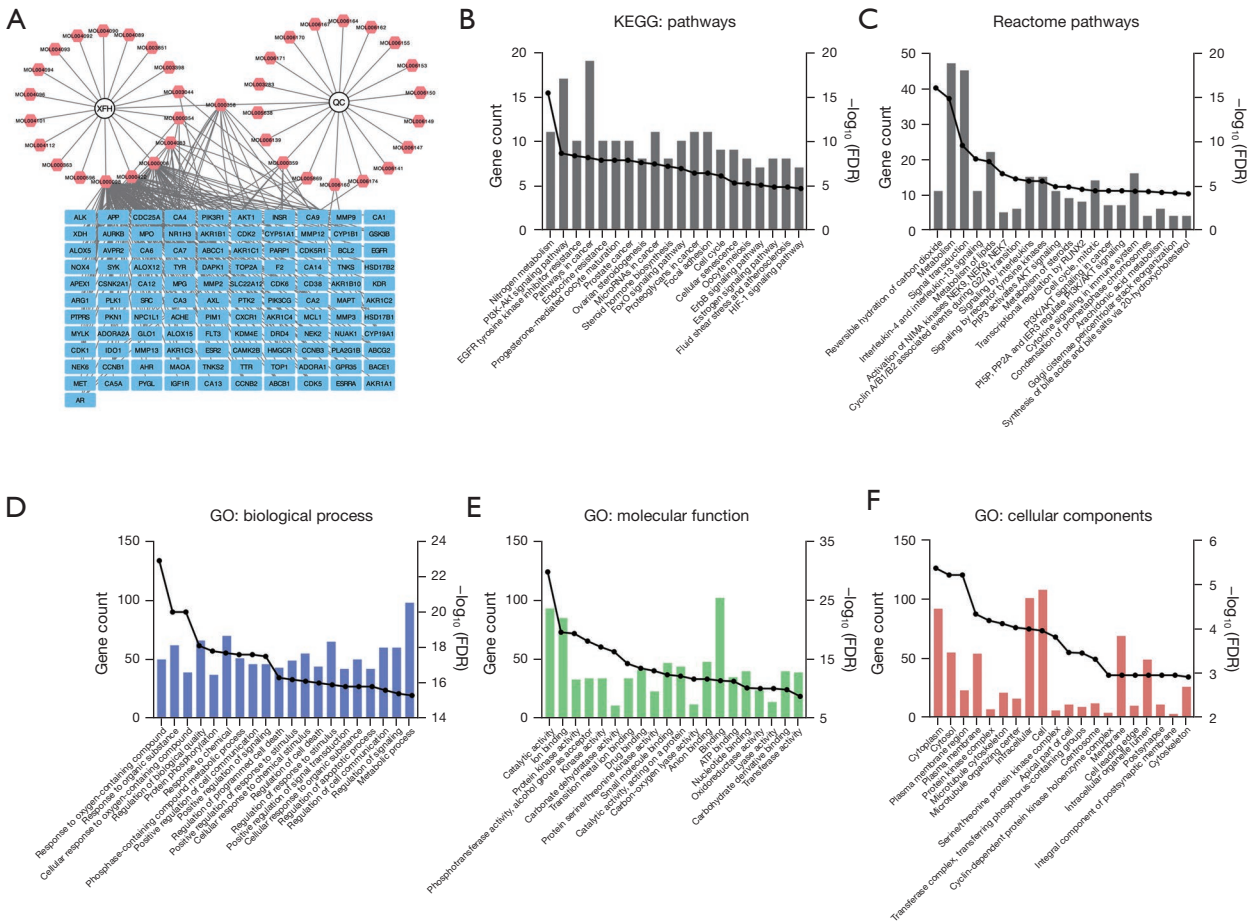

Figure 4 Network pharmacological analysis of Xuan-Fu-Hua decoction effective components and their putative target proteins. (A) The interaction network consisting of Xuan-Fu-Hua decoction effective components and their putative target proteins was constructed using Cytoscape software. (B-F) Gene set enrichment analysis results of the genes corresponding to the putative target proteins of Xuan-Fu-Hua decoction effective components. The top 20 most significantly enriched terms in each set are presented. Columns represent the number of genes from the differentially expressed genes that were mapped to each term, and the dotted line indicates the ranking of –log10 (FDR) value. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; FDR, false discovery rate.

lung diseases, which is simple in components and have mild temperament. Zhao et al. reported in a previous study that treatment with Xuan-Fu-Hua extract could attenuate bleomycin-induced murine pulmonary fibrosis and systemic inflammation (10). In a study on the effects of Xuan-Fu-Hua decoction on serum inflammatory indexes and T lymphocyte subset of patients with chronic atrophic gastritis (CAG), the levels of TNF-α, IL-6 and IL-8 in patients treated with Xuan-Fu-Hua decoction were significantly lower than before and in control group, while CD3+, CD4+ and CD4+/CD8+ were significantly increased. Namely, Xuan-Fu-Hua decoction could reduce the level of serum inflammatory factors in CAG patients, regulate the immune function, and then improve the clinical treatment effect (11). Xuan-Fu-Hua decoction has been used in the management of liver cancer in some clinical trials, and are no liver/renal dysfunction caused by Xuan-Fu-Hua decoction in literature or clinical practice, yet its antitumor effect and mechanism of action have not been evaluated in biomedical research before. In modern Traditional Chinese Medicine (TCM) clinical practice, the common formula for Xuan-Fu-Hua decoction is Xuan-Fu-Hua, Qian-Cao, and Cong-Bai. In preliminary research we compared the effect of Xuan-Fu-Hua decoction with or without Cong-Bai for the treatment of xenograft tumor established by liver cancer cells on nude mice, and the results suggested that Cong-Bai seemed

dispensable for the antitumor property of Xuan-Fu-Hua decoction. Therefore, in this research, we primarily focused on investigating the therapeutic effect and mechanism of action of Xuan-Fu-Hua and Qian-Cao. We observed, for the first time, that treatment with Xuan-Fu-Hua decoction could significantly augment the antitumor effect of 5-FU on liver cancer in both *in vitro* and *in vivo* settings, rather than inhibiting tumor growth by itself. Since 5-FU inhibits tumor progression primarily through its cytotoxic effects, we speculated that Xuan-Fu-Hua decoction treatment might enhance the effect of 5-FU treatment by potentiating liver cancer cells to chemically induce cell apoptosis either by augmenting the proapoptotic machinery or repressing the antiapoptotic, prosurvival mechanism.

We then compared the transcriptome of SMMC-7721 liver cancer cells with or without Xuan-Fu-Hua decoction treatment to unravel its impact in detail. Through enrichment analysis of the genes with significant differential expression induced by Xuan-Fu-Hua decoction treatment, we found that Xuan-Fu-Hua decoction treatment mainly affected the transcription of genes involved in the cell apoptosis process. Among these genes, we found that the transcription levels of *DDIT3*, *PMAIP1*, and *ZMAT3* genes were significantly increased by Xuan-Fu-Hua decoction treatment, while that of *WNT4*, *AXIN2*, *NFE2L2*, *TGFBR1*, *MITF*, and *IGFBP3* genes were significantly downregulated by Xuan-Fu-Hua decoction treatment. The CCAAT/enhancer-binding homologous protein (CHOP) encoded by the *DDIT3* gene can be activated in the cytosol by ATF4 and directly inhibit the activity of antiapoptotic protein Bcl-2 and Bcl-xl on the mitochondrial membrane (12-15), while the Phorbol-12-myristate-13-acetate-induced protein 1 (previously termed Noxa) encoded by the *PMAIP1* gene participates in the cytochrome c release from the mitochondria (16,17). Zinc finger matrin-type protein 3, encoded by the *ZMAT3* gene, was recently found essential for the p53-dependent suppression of tumor growth (18,19). On the other hand, the significant downregulation in *TGFBR1* gene transcription might have reduced TGF-beta receptor 1 expression and its downstream TGF-beta signaling pathway, which has been found to facilitate the therapeutic resistance of liver cancer cells (20). Downregulation in *WNT4* (21,22) and *AXIN2* (23-25) gene transcription might have also impaired the activation of beta-catenin and its antiapoptotic role in liver cancer cells. The nuclear factor erythroid 2 like 2 protein encoded by the *NFE2L2* (also known as *NRF2*) gene has been recognized as a cancer-promoting gene due to its role in regulating the transcription of its target genes implicated in cancer cell metabolism (26), and the activation of this transcription factor protects liver cancer cells from ferroptosis (27). Collectively, the results of transcriptomic analysis suggested that Xuan-Fu-Hua decoction treatment augmented the proapoptotic machinery and repressed the prosurvival mechanism in liver cancer cells, possibly enhancing the cytotoxicity of 5-FU treatment as a result. It suggests that Xuan-Fu-Hua decoction may be used as an adjunct to chemotherapy drugs in the treatment of liver cancer.

It is generally accepted that the effective components from TCM or other natural products exert their medicinal effects mainly by binding to biomolecules within cells and consequently influencing the biological functions of the latter. Since biomolecules depend on a specific molecular structure to perform their biological functions within the cell, and the binding of other molecules to them often has an impact on their molecular structure, the binding of natural product effective components to biomolecules often inhibits the biological functions of the latter, such as the catalytic activity of enzymes bound to substrates or the transcriptional activity of transcription factors recognizing specific chromatin sites. To investigate how Xuan-Fu-Hua decoction treatment regulated the sensitivity of liver cancer cells to the cytotoxicity of 5-FU, we employed SwissTargetPrediction to explore the biomolecules possibly interacting with Xuan-Fu-Hua decoction effective components. We found that MOL000098 (Quercetin) in Xuan-Fu-Hua might interact with multiple receptor tyrosine kinases as well as PIK3CG, PIK3R1, and AKT1 in the PI3K/AKT signaling pathway, and we found that MOL006160 (Alizarin) in Qian-Cao might interact with Bcl-2 and Mcl-1, the apoptosis-inhibiting and tumor growth-favoring roles of which have been well established. We speculated that Xuan-Fu-Hua decoction treatment sensitized liver cancer cells to 5-FU treatment possibly by inhibiting the activation of the PI3K/AKT signaling pathway and the Bcl-2/Mcl-1 proteins. Considering the implication of the hyperactivation of PI3K/AKT signaling in the resistance of receptor tyrosine kinas inhibitor treatment in liver cancer and many other types of tumors, Xuan-Fu-Hua decoction might also improve the therapeutic effect of this targeted therapy. Further studies are needed to determine whether Quercetin and Alizarin are the main effective components in the antitumor activity of Xuan-Fu-Hua decoction.

The original recipe for Xuan-Fu-Hua decoction was

recorded in the "Jin-Kui-Yao-Lue (Synopsis of Prescriptions of the Golden Chamber)" as Xuan-Fu-Hua, "Xin-Jiang", and Cong-Bai. Due to a lack of documentary and historical evidence, there is still no definitive answer to the question of what kind of medicine "Xin-Jiang" is, but it is generally believed to refer to cloth freshly dyed with dye made from Qian-Cao. It has been found in clinical practice that the use of Qian-Cao in the Xuan-Fu-Hua decoction is effective. In this study, our network pharmacology results showed that Alizarin was the only effective component in Qian-Cao with the highest interaction probability with its putative target proteins Bcl-2 and Mcl-1. Alizarin has long been used as a natural pigment in the weaving and dyeing industry, seemingly suggesting it as the major effective component in Qian-Cao.

Conclusions

Overall, our data showed that cotreatment with Xuan-Fu-Hua decoction significantly sensitized SMMC-7721 liver cancer cells to 5-FU treatment-induced growth inhibition and apoptosis, possibly by inhibiting the activation of PI3K/AKT signaling and the transcription of the downstream prosurvival genes by inhibiting the antiapoptotic function of Bcl-2 and Mcl-1 proteins and by increasing the transcription of proapoptotic genes. Xuan-Fu-Hua decoction might be useful in adjuvant therapy for liver cancer treatment to improve patient outcomes.

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Footnote

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References

1. Bhagyaraj E, Ahuja N, Kumar S, et al. TGF-β induced chemoresistance in liver cancer is modulated by xenobiotic nuclear receptor PXR. Cell Cycle 2019;18:3589-602.
2. Huang X, Gan G, Wang X, et al. The HGF-MET axis coordinates liver cancer metabolism and autophagy for chemotherapeutic resistance. Autophagy 2019;15:1258-79.
3. Wang Y, Huang P, Hu Y, et al. An oncolytic adenovirus delivering TSLC1 inhibits Wnt signaling pathway and tumor growth in SMMC-7721 xenograft mice model. Acta Biochim Biophys Sin (Shanghai) 2021;53:766-74.
4. Chon HJ, Kim C. REACH-2: first biomarker-based anti-angiogenic therapy in patients with advanced hepatocellular carcinoma. Chin Clin Oncol 2020;9:58.
5. Xiong L. Laboratory study on antiliver fibrosis and antisinusoid capillarization of Xuanfuhua decoction. Chinese Journal of Traditional Medical Science and Technology 1999;6:20-1.
6. Liu LH, Wang T, Zhou G, et al. Experience of Professor WEI Zhong-min in Treating Internal Diseases by Application of Xuanfuhua Decoction. Chinese Journal of Information on Traditional Chinese Medicine 2017;24:101-3.
7. Li S, Zhang B. Traditional Chinese medicine network pharmacology: theory, methodology and application. Chin J Nat Med 2013;11:110-20.
8. Zhang R, Zhu X, Bai H, et al. Network Pharmacology

Databases for Traditional Chinese Medicine: Review and Assessment. Front Pharmacol 2019;10:123.
9. Gao L, Cao M, Li JQ, et al. Traditional Chinese Medicine Network Pharmacology in Cardiovascular Precision Medicine. Curr Pharm Des 2021;27:2925-33.
10. Zhao WY, Luan ZL, Liu TT, et al. Inula japonica ameliorated bleomycin-induced pulmonary fibrosis via inhibiting soluble epoxide hydrolase. Bioorg Chem 2020;102:104065.
11. Chen K, Guan XM. Clinical effects of Modified Xuanfuhua Decoction and Wendan Decoction on patients with chronic atrophic gastritis due to Phlegm-Blood Stasis Blocking Collaterals. Modern Journal of Integrated Traditional Chinese and Western Medicine 2022;31:1124-1127.
12. Li Y, Jiang W, Niu Q, et al. eIF2α-CHOP-BCl-2/JNK and IRE1α-XBP1/JNK signaling promote apoptosis and inflammation and support the proliferation of Newcastle disease virus. Cell Death Dis 2019;10:891.
13. Gaudette BT, Iwakoshi NN, Boise LH. Bcl-xL protein protects from C/EBP homologous protein (CHOP)-dependent apoptosis during plasma cell differentiation. J Biol Chem 2014;289:23629-40.
14. Li D, Wang WJ, Wang YZ, et al. Lobaplatin promotes 125I-induced apoptosis and inhibition of proliferation in hepatocellular carcinoma by upregulating PERK-eIF2α-ATF4-CHOP pathway. Cell Death Dis 2019;10:744.
15. Shen J, Su Z. Vanillin oxime inhibits lung cancer cell proliferation and activates apoptosis through JNK/ERK-CHOP pathway. Korean J Physiol Pharmacol 2021;25:273-80.
16. Delannoy A, Wilhelm E, Eilebrecht S, et al. BIM and NOXA are mitochondrial effectors of TAF6δ-driven apoptosis. Cell Death Dis 2018;9:70.
17. Roufayel R, Younes K, Al-Sabi A, et al. BH3-Only Proteins Noxa and Puma Are Key Regulators of Induced Apoptosis. Life (Basel) 2022;12:256.
18. Bieging-Rolett KT, Kaiser AM, Morgens DW, et al. Zmat3 Is a Key Splicing Regulator in the p53 Tumor Suppression Program. Mol Cell 2020;80:452-469.e9.
19. Janic A, Valente LJ, Wakefield MJ, et al. DNA repair processes are critical mediators of p53-dependent tumor suppression. Nat Med 2018;24:947-53.
20. Wang B, Xu X, Yang Z, et al. POH1 contributes to hyperactivation of TGF-β signaling and facilitates hepatocellular carcinoma metastasis through deubiquitinating TGF-β receptors and caveolin-1. EBioMedicine 2019;41:320-32.
21. Huang Z, Yang M, Li Y, et al. Exosomes Derived from Hypoxic Colorectal Cancer Cells Transfer Wnt4 to Normoxic Cells to Elicit a Prometastatic Phenotype. Int J Biol Sci 2018;14:2094-102.
22. Yang D, Li Q, Shang R, et al. WNT4 secreted by tumor tissues promotes tumor progression in colorectal cancer by activation of the Wnt/β-catenin signalling pathway. J Exp Clin Cancer Res 2020;39:251.
23. Katoh M. Multi-layered prevention and treatment of chronic inflammation, organ fibrosis and cancer associated with canonical WNT/β-catenin signaling activation (Review). Int J Mol Med 2018;42:713-25.
24. Li Y, Jin K, van Pelt GW, et al. c-Myb Enhances Breast Cancer Invasion and Metastasis through the Wnt/β-Catenin/Axin2 Pathway. Cancer Res 2016;76:3364-75.
25. Sun T, Annunziato S, Bergling S, et al. ZNRF3 and RNF43 cooperate to safeguard metabolic liver zonation and hepatocyte proliferation. Cell Stem Cell 2021;28:1822-1837.e10.
26. Rojo de la Vega M, Chapman E, Zhang DD. NRF2 and the Hallmarks of Cancer. Cancer Cell 2018;34:21-43.
27. Sun X, Ou Z, Chen R, et al. Activation of the p62-Keap1-NRF2 pathway protects against ferroptosis in hepatocellular carcinoma cells. Hepatology 2016;63:173-84.

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Supplementary

Table S1 Effective components of Inulae Flos (Xuan-Fu-Hua) and Rubia cordifolia (Qian-Cao) in Xuan-Fu-Hua decoction documented in TCMSP database with OB higher than 30% and DL index greater than 0.18

Ingredient	Molecule name	Pubchem cid	Mol ID	MW	OB (%)	DL
Inulae Flos (Xuan-Fu-Hua)	luteolin	5280445	MOL000006	286.25	36.16	0.25
	quercetin	5280343	MOL000098	302.25	46.43	0.28
	isorhamnetin	5281654	MOL000354	316.28	49.6	0.31
	beta-sitosterol	222284	MOL000358	414.79	36.91	0.75
	amyrin Palmitate (β-amyrin palmitate)	13915599	MOL000363	665.26	32.68	0.3
	kaempferol	5280863	MOL000422	286.25	41.88	0.24
	[(3S,4aR,6aR,6aR,6bR,8aR,12S,12aR,14aR,14bR)-4,4,6a,6b,8a,12,14b-heptamethyl-11-methylene-1,2,3,4a,5,6,6a,7,8,9,10,12,12a,13,14,14a-hexadecahydropicen-3-yl] acetate	13889352	MOL000596	468.84	43.08	0.74
	Chryseriol	5280666	MOL003044	300.28	35.85	0.27
	Pratensein	5281803	MOL003398	299.27	39.06	0.28
	Isoramanone (digipurpurogenin II)	68807316	MOL003851	348.53	39.97	0.51
	Tamarixetin	5281699	MOL004083	316.28	32.86	0.31
	inulicin	442263	MOL004089	308.41	30.12	0.22
	3-[(3aS,4R,5R,8aR)-4-hydroxy-5,7-dimethyl-3-methylene-2-oxo-4,5,8,8a-tetrahydro-3aH-cyclohepta[b]furan-6-yl]propyl acetate	36564	MOL004090	308.41	73.35	0.22
	[(3aR,4R,7aR)-5-[(1S)-4-acetoxy-1-methyl-butyl]-6-methyl-3-methylene-2-oxo-3a,4,7,7a-tetrahydrobenzofuran-4-yl] acetate	10360513	MOL004092	350.45	39.03	0.31
	Azaleatin	5281604	MOL004093	316.28	54.28	0.3
	Britanin	5315501	MOL004094	366.45	33.73	0.41
	epifriedelanol acetate	13688752	MOL004096	470.86	31.18	0.74
	Melilotoside	5280759	MOL004101	326.33	36.85	0.26
	Patuletin	5281678	MOL004112	332.28	53.11	0.34
Rubia Cordifolia (Qian-Cao)	beta-sitosterol	222284	MOL000358	414.79	36.91	0.75
	sitosterol	12303645	MOL000359	414.79	36.91	0.75
	(2R,3R,4S)-4-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dimethylol-tetralin-6-ol	160521	MOL003283	360.44	66.51	0.39
	Mollugin	124219	MOL005638	284.33	42.34	0.26
	daucostero_qt	N/A	MOL005869	414.79	36.91	0.75
	1,3-dimethoxy-2-carboxyanthraquinone	N/A	MOL006139	312.29	102.89	0.33
	1,3-dihydroxy-2-hydroxymthylanthraquinone-3-O-xylosyl(1→6)-glucoside_qt	N/A	MOL006141	284.28	71.27	0.27
	Alizarin-2-methylether	80103	MOL006147	254.25	32.81	0.21
	7-hydroxy-8-methyl-4-vinyl-9,10-dihydrophenanthrene-1-carboxylic acid	9993796	MOL006149	278.32	56.99	0.27
	1-acetoxy-6-hydroxy2-methylanthraquinone-3-O-α-rhamnosyl(1→4)-α-glucoside	N/A	MOL006150	616.67	30.74	0.64
	2'-hydroxymollugin	N/A	MOL006153	302.35	40.5	0.29
	4-hydroxy-9,10-dioxoanthracene-2-carboxylic acid	179447	MOL006155	266.21	45.98	0.25
	Alizarin	6293	MOL006160	240.22	32.67	0.19
	Nordamnacanthal	160712	MOL006162	268.23	53.97	0.24
	Pallasone	5318483	MOL006164	374.62	43.87	0.4
	methyl 6-hydroxy-2,2-dimethyl-3,4-dihydrobenzo[h]chromene-5-carboxylate	10779560	MOL006167	286.35	51.09	0.25
	Henine	10163	MOL006170	270.25	77.12	0.24
	rubiprasin B	N/A	MOL006171	498.87	35.97	0.68
	Xyloidone	72734	MOL006174	240.27	31.61	0.18

OB, oral bioavailability; DL, drug-like; MW, molecular weight; N/A, the molecule has no Pubchem Cid.

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