J Appl Biomed
DOI: 10.32725/jab.2023.017
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

The molecular targets of Kangai injection in gastric cancer by *in silico* network pharmacology approach and experiment confirmation

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Abstract

Introduction: This study aimed to identify the phytochemical constituents that could target gastric cancer in Kangai injection using a network pharmacology-based approach.

Methods: Protein-protein interactions (PPI), Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted utilizing String and OmicShare tools. In the *in vitro* experiments, the related mRNA and protein levels were assessed via real-time quantitative polymerase chain reaction and Western blotting, respectively. Cell proliferation was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay.

Results: Kangai injection comprises several compounds, which target multiple substrates and pathways related to gastric cancer. The PPI and Gene Ontology analyses revealed that tumor necrosis factor (*TNF*) was a hub gene. KEGG pathway enrichment analysis indicated that the the TNF pathway was significantly enriched. Kangai injection decreased the mRNA levels of TNFR2, TRAF2, PI3K, AKT, and IκBα and inhibited the phosphorylation of PI3K, AKT, and IκBα phosphorylations. Kangai injection inhibited cell proliferation, while TNFR2 overexpression or treatment with the PI3K activator 740 Y-P partially restored it.

Conclusion: Kangai injection operates through multiple targets and pathways in gastric cancer, with the TNFR2/PI3K/AKT/NF-κB pathway playing a crucial role in its mechanism against gastric cancer.

Keywords: Gastric cancer; Kangai injection; Network pharmacology; TNF pathway

Highlights:

- The PPI network analysis and Gene Ontology enrichment analysis indicated the hub role of TNF.
- Kangai injection represents multi-target and multi-pathway mechanisms in gastric cancer.
- TNFR2/PI3K/AKT/NF-κB pathway involved in the mechnism of Kangai injection against gastric cancer.

Introduction

The global prevalence of gastric cancer is approximately 990,000 cases annually (Smyth et al., 2020). This number is expected to increase due to an aging population (Machlowska et al., 2020). Gastric cancer has high mortality rates, often resulting in poor survival outcomes (Orman and Cayci, 2019; Thrift and El-Serag, 2020). A significant percentage of these cases arise in Asia, particularly in China (Endo et al., 2022). Gastric cancer results from complex interactions between individual and environmental factors (Yusefi et al., 2018). Different subtypes of this disease exhibit distinct epidemiological and pathological traits (Joshi and Badgwell, 2021). The treatment strategy is selected based on the tumor stage, with chemotherapy commonly used for metastatic or inoperable cases.

Several cytotoxic agents such as fluoropyrimidines, platin-based derivatives, and taxanes are active in advanced forms of gastric cancer, being used as combination therapy to obtain higher response rates (Song et al., 2017). However, their side effects drive many patients towards natural herbal remedies, which may also be more economical (Tabassam et al., 2021; Zhao et al., 2023).

The benefits of herbs has been increasingly acquainted in recent years (Abdulridha et al., 2020). Based on the data from the world health organization, a large population of the world tends to select traditional treatment methods (Khan et al., 2019). It would be beneficial to clarify the potential mechanism underlying the effects of molecules from herbs or formulas in diseases using modern biomolecular technology. For example, resveratrol and curcumin have been proven as anticancer molecules in gastric cancer (Hassanalilou et al., 2019; Zulueta et al., 2015). The anticancer effect of resveratrol is

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http://doi.org/10.32725/jab.2023.017

Submitted: 2023-01-31 • Accepted: 2023-09-14 • Prepublished online: 2023-09-22
J Appl Biomed 21/3: 150–159 • EISSN 1214-0287 • ISSN 1214-021X

mediated through various molecular signaling pathways, such as Hedgehog, Wingless-related integration site/β-catenin, or phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB or AKT)/ phosphorylated (p)-Forkhead box protein O4 (FoxO4) signaling pathways (Ashrafizadeh et al., 2021). This highlights the inherent multi-target capabilities of herbs. Kangai injection, a mix of *Hedysarum multijugum* Maxim (HMM), *Panax ginseng* C. A. Mey, and matrine, has proven effective in advanced stages of non-small cell lung cancer, advanced colorectal cancer, and breast cancer (Huang et al., 2019; Li et al., 2019; Xue et al., 2018). In gastric cancer, combining Kangai injection with folinic acid, fluorouracil, and oxaliplatin (FOLFOX)-based chemotherapy resulted in a higher clinical efficacy than using only FOLFOX (Zhang et al., 2017). However, the exact mechanism underlying the effect of Kangai injection in gastric cancer remains under-researched.

The aim of this study was to elucidate this mechanism by employing a network-pharmacology approach. First, active components were isolated, and their targets were identified. Subsequently, potential target genes related to gastric cancer were selected. By comparing the genes targeted by both Kangai injection and those linked to gastric cancer, a Gene Ontology (GO) terms classification and pathway enrichment analysis were undertaken. To confirm the obtained results, cell experiments were conducted.

Materials and methods

Filtration in chemical database

Phytochemicals of *Hedysarum multijugum* Maxim (HMM) and *Panax ginseng* C. A. Mey (PG) in Kangai injection have been retrieved from the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP) databank (https://old.tcmsp-e.com/tcmspsearch.php?qr=Trichosant-his%20Radix&qsr=herb_en_name&token=5e71a774a2ba476dc090a8917505d8d9). The following parameters were used to filter the most relevant compounds: oral bioavailability (OB) ≥30%, drug-likeness (DL) ≥0.18, and fractional water accessible surface area (FASA) <0.339. These parameters are standard for assessing the potential of bioactive molecules as therapeutic agents. Matrine comprises approximately 98% oxymatrine; therefore oxymatrine was directly included in the analysis.

Collection of genes related to the active ingredients

The target genes for the phytochemicals of HMM and *Panax ginseng* C. A. Mey in Kangai injection were identified and collected from the TCMSP. The target genes for oxymatrine in matrine were obtained from SymMap (http://www.symmap.org/). The plant-compound-gene network was assembled using Cytoscape 3.7.1.

Identification of gastric cancer-related gene

Using the GeneCards® human gene database (https://www.genecards.org/) and the Therapeutic Target Database (TTD, http://db.idrblab.net/ttd/), as well as the search term "gastric cancer", we searched for genes related to gastric cancer.

Acquisition of the overlapping genes

To unify gene names from different sources, the UniProt database was employed. Subsequently, the Venny 2.1.0 online tool was utilized to produce Venn diagrams of the related target genes.

Prediction for protein-protein interaction (PPI) networks

Proteins corresponding to the overlapping genes were identified, and a protein-protein interaction (PPI) network was constructed using the STRING database. For clustering analysis, the K-means clustering method was applied with a set cluster count of five.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

Evaluation of the overlapping genes was carried out using Gene Ontology and KEGG pathway enrichment analyses via the OmicShare 6.4.5 online tool. The former provides a dynamically updated standard vocabulary to comprehensively describe gene properties. The latter, using KEGG as a reference, employs a hypergeometric test to identify pathways significantly enriched in the selected genes relative to the entire genome (Castaneda et al., 2023).

Cell culture and treatment

Kangai injection provided by Changbaishan Pharmaceutical Co., Ltd. (Jilin, China) was analyzed. It met the quality standards set by the Chinese National Medical Products Administration (WS-11222[ZD-1222]-2002). Human gastric cancer cell lines HGC-27 and NCI-N87, obtained from the Chinese National Collection of Authenticated Cell Cultures, were cultured in a medium comprising 90% Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO Life Technologies, USA) and 10% fetal bovine serum (GIBCO, Australia).

The cells were allowed to grow until they reached logarithmic growth phase. Subsequently, they were seeded in 96-well plates at a density of 6×10^3 per well. Two groups were established: a control group (Con) without Kangai treatment and a group treated with Kangai. The employed drug concentrations were 300 μl × ml^{-1} (corresponding to 3.0 mg × ml^{-1} of matrine). Each experiment was performed in triplicate. Cultivation was done overnight at 37 °C in a 5% CO_2 incubator. A Tumor Necrosis Factor Receptor 2 (*TNFR2*) over-expression vector (ov-TNFR2) was purchased from Shanghai GenePharma (Shanghai, China).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay

MTT assay kits (Abcam, ab211091, USA) were purchased to assess cell proliferation. The cells were transfected with ov-TNFR2 using Lipofectamine 2000 (Invitrogen, USA) or treated with 740 Y-P, and then incubated in media containing a concentration of Kangai injection for 24, 48, and 72 hours. At each time point, the serum-containing media was replaced with serum-free media containing the MTT reagent. An additional 3-hour incubation was required before measuring absorbance at 590 nm using a microplate reader.

Real-time quantitative polymerase chain reaction (RT-qPCR) analyses

HGC-27 cells (5×10^6) treated by Kangai injection for 48 hours were harvested. Total RNA was extracted using the MiniBEST Universal RNA Extraction Kit (Takara, Japan). RNA purity was assessed by measuring the A260/A280 ratio using a NanoDrop (ThermoScientific, USA) and RNA quality checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Total extracted RNA (500 ng) was subjected to reverse transcription using the PrimeScript RT Master Mix kit (Takara, Japan). RT-qPCR was performed using the SYBR Premix Ex TaqTMII

kit (Takara, Japan) on a Bio-Rad iQ5 PCR cycler (USA). β-actin served as the internal reference. The parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. All experiments were performed in triplicate. Relative levels of genes or RNAs were normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method (Table 1).

Table 1. qPCR primer sequences

Target gene	Primer sequence (forward)	Primer sequence (reverse)
TNFR2	ACAGTGCCCGCCCAGGTTGTCTTG	GCAGAAATGTTTCACATATTGGCCAGGAGG
TRAF2	CACCGGTACTGCTCCTTCTG	TGAACACAGGCAGCACAGTT
PI3K	CTTGCCTCCATTCACCACCTCT	GCCTCTAATCTTCTCCCTCTCCTTC
AKT	TGTCTCGTGAGCGCGTGTTTTT	CCGTTATCTTGATGTGCCCGTC
IκBα	TGAAGGACGAGGAGTACGAGC	TGCAGGAACGAGTCTCCGT
β-actin	5′-GCTTCTTTGCAGCTCCTTCGT-3′	5′-CCTTCTGACCCATTCCCACC-3′

Western blot

Treated NCI-N87 cells (5×10^5) were harvested and lysed using RIPA buffer (Thermo Scientific, USA) for 10 min at 4 °C. BSA Standard Solution (2 mg/ml) from BCA Protein Assay Kit (Tiangen, China) was diluted at a gradient concentration for standard curve construction according to the instructions. The blotting process was performed at eZwest Lite Auto Western Blotting System (Nanjing, China). Equivalent amount of protein (20 μg) was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked with skimmed milk (5%) for 1 h. Then, the membranes were incubated with the primary antibodies against TNFR2 (catalog #3727, Cell signaling, USA), TRAF2 (#4712, Cell signaling), PI3K (#4255, Cell signaling), p-PI3K (#4228S, Cell signaling), AKT (#4060, Cell signaling), p-AKT (#4060, Cell signaling) , IκBα, and p-IκBα (all 2 μg/ml), overnight at 4 °C, with β-actin (clone #SP124, Abcam, USA) as control. Then, biotinylated goat anti-rabbit IgG (#A0277; 2 μg/ml; Beyotime Biotech) was added. Horseradish peroxidase-streptavidin and an ECL kit (Thermo Scientific, Waltham, MA, USA) were used for color rendering. The band intensities were quantified using Image J software. The quantification of each immunoblot was performed on three biological repeats.

Statistical analysis

Data were obtained from several independent experiments. The results are presented as mean ± standard deviation (SD). For statistical significance analysis, we applied a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or two-way ANOVA and Sidak's multiple comparison test. Differences were considered statistically significant for P levels < 0.05.

Results

Phytochemical components in Kangai injection

A total of 279 compounds were obtained from TCMSP (190 for *Panax ginseng* C. A. Mey., 87 for *Hedysarum multijugum* Maxim, and 2 for marine). OB represents the percentage of a drug that reaches the systemic circulation; FASA- can be used as a drug-likeness evaluation for drug-like molecules; DL is a qualitative concept for an estimate on how "drug-like" a prospective compound is. According to their OB, FASA- and DL values, fourteen candidate compounds from *Panax ginseng* C. A. Mey., and fourteen from *Hedysarum multijugum* Maxim were screened. Combined with the main compound of marine (oxymatrine), a total of 29 phytochemicals were listed in Table 2.

Table 2. Information for active chemical compounds of Kangai injection

Compositions of Kangai injection	Mol ID	Molecule name	OB (%)	DL	FASA-
Hedysarum multijugum Maxim	MOL000211	Mairin	55.38	0.78	0.26
	MOL000239	Jaranol	50.83	0.29	0.29
	MOL000296	hederagenin	36.91	0.75	0
	MOL000033	(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R,5S)-5-propan-2-yloctan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	36.23	0.78	0
	MOL000354	isorhamnetin	49.60	0.31	0.32
	MOL000371	3,9-di-O-methylnissolin	53.74	0.48	0
	MOL000378	7-O-methylisomucronulatol	74.69	0.30	0
	MOL000379	9,10-dimethoxypterocarpan-3-O-β-D-glucoside	36.74	0.92	0
	MOL000380	(6aR,11aR)-9,10-dimethoxy-6a,11a-dihydro-6H-benzofurano[3,2-c]chromen-3-ol	64.26	0.42	0
	MOL000387	Bifendate	31.10	0.67	0
	MOL000392	formononetin	69.67	0.21	0

Table 2. ***(continued)***

Compositions of Kangai injection	Mol ID	Molecule name	OB (%)	DL	FASA-
Hedysarum multijugum Maxim	MOL000417	Calycosin	47.75	0.24	0
	MOL000422	kaempferol	41.88	0.24	0
	MOL000433	FA	68.96	0.71	0
	MOL000442	1,7-Dihydroxy-3,9-dimethoxy pterocarpene	39.05	0.48	0
Panax ginseng C. A. Mey	MOL002879	Diop	43.59	0.39	0.28
	MOL000449	Stigmasterol	43.83	0.76	0.22
	MOL000358	beta-sitosterol	36.91	0.75	0.23
	MOL003648	Inermin	65.83	0.54	0.30
	MOL000422	kaempferol	41.88	0.24	0
	MOL005317	Deoxyharringtonine	39.27	0.81	0.23
	MOL005320	arachidonate	45.57	0.20	0.26
	MOL005344	ginsenoside rh2	36.32	0.56	0.24
	MOL005348	Ginsenoside-Rh4_qt	31.11	0.78	0.25
	MOL005356	Girinimbin	61.22	0.31	0.33
	MOL005376	Panaxadiol	33.09	0.79	0.22
	MOL005384	suchilactone	57.52	0.56	0.28
	MOL005399	alexandrin_qt	36.91	0.75	0.23
	MOL000787	Fumarine	59.26	0.83	0.30
Marine	MOL006634	Oxymatrine	0.35	0.28	0.03

Construction of the plant-compound-gene network

The 29 candidate compounds were matched to 148 human target genes in the TCMSP database (Suppl. 1). This relationship between the compounds and target genes was represented in a compound-target protein network (Fig. 1). The network comprised 181 nodes (3 for herbs, 30 for compounds, and 148 for target genes) and 574 connecting edges. Each compound targeted, on average, 4.9 (148/30) genes, while each gene was associated with approximately 3.9 (574/148) compounds. This indicates that Kangai injection is a multi-component formulation and targets multiple substrates. The average degree of the nodes in the network was 6.3, with 36 nodes exceeding this average. The average betweenness centrality of nodes was 0.0117, with 42 nodes surpassing this value.

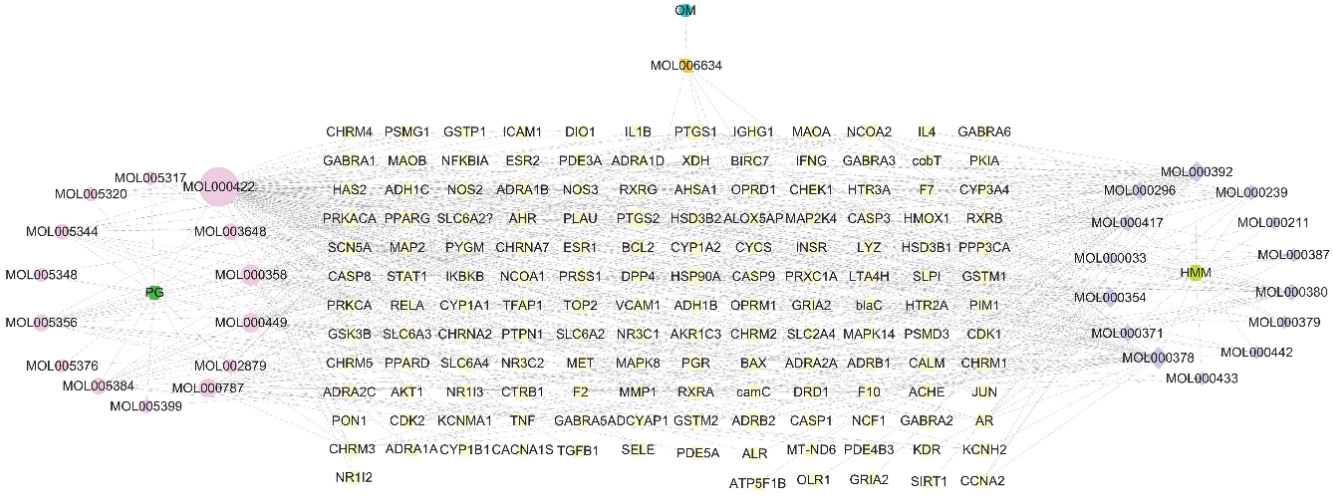

Fig. 1. Network of compound-target gene interactions (Cytoscape 3.7.1) for Kangai injection. PG, *Panax ginseng* C. A. Mey. HMM, *Hedysarum multijugum* Maxim. OM: Marine. The yellow node represents the target genes of Kangai injection, the pink nodes represent core active compounds of PG, the purple node represents core active compounds of HMM, and the orange nodes represent core active compounds of Marine. The size of the nodes is positively related to their degree.

Construction of key protein network of Kangai injection and gastric cancer

To further explore the mechanism underlying the effect of Kangai injection on gastric cancer, we collected the gastric cancer-related genes from Genecards and the TTD. By cross-referencing phytochemical-targeted with gastric cancer-associated genes using Venny 2.1, we identified the overlapping genes. As shown in Fig. 2A, out of these, 123 phytochemical-targeted genes were also related to gastric cancer. These 123 genes were subjected to analysis in the String database, with a confidence threshold set to exceed 0.9. The resulting PPI networks, categorized through K-means clustering, are illustrated in Figs 2B–2F. The PPI network of overlapping genes comprised 123 nodes and 1,136 edges, exhibiting an average degree of 18.5. The 20 most significant genes are listed in Table 3, with AKT and TNF ranked as the top two.

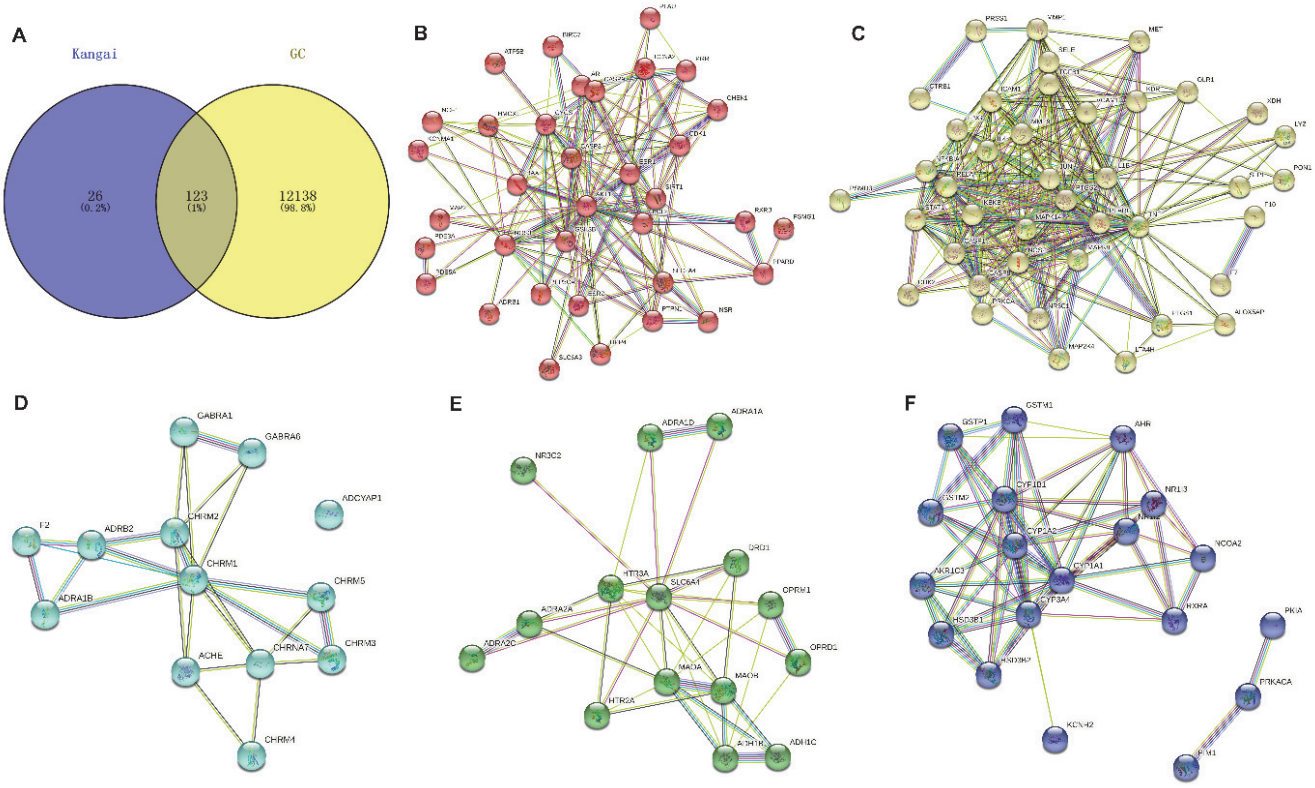

Fig. 2. Construction of protein-protein interactions (PPI) network (**A**) Venn diagram of the overlapping genes from Kangai injection-related and gastric cancer (GC)-related genes. A total of 123 overlapping genes were obtained. (**B–F**) The PPI network of the potential targets associated with Kangai injection related to gastric cancer with K-means clustering ($P < 1.0 \times 10^{-16}$).

Table 3. The top 20 hub genes in the PPI network

#Node	Identifier	Node_degree
AKT1	9606.ENSP00000451828	68
TNF	9606.ENSP00000398698	64
JUN	9606.ENSP00000360266	57
CASP3	9606.ENSP00000311032	53
IL1B	9606.ENSP00000263341	52
ESR1	9606.ENSP00000405330	51
PTGS2	9606.ENSP00000356438	51
PPARG	9606.ENSP00000287820	49
MMP9	9606.ENSP00000361405	47
NOS3	9606.ENSP00000297494	44
NFKBIA	9606.ENSP00000216797	41
SIRT1	9606.ENSP00000212015	41
RELA	9606.ENSP00000384273	39
CYCS	9606.ENSP00000307786	37
HMOX1	9606.ENSP00000216117	37
MAPK14	9606.ENSP00000229795	37
MAPK8	9606.ENSP00000378974	36
CASP8	9606.ENSP00000351273	35
STAT1	9606.ENSP00000354394	34
GSK3B	9606.ENSP00000324806	32

Pharmacological mechanisms underlying the effect of Kangai injection acting on gastric cancer

To gain further insight into the mechanism underlying the effect of Kangai injection on gastric cancer, we conducted Gene Ontology and KEGG pathway enrichment analyses. Through Gene Ontology enrichment analysis, 460 biological processes were identified, with 130 being significantly enriched ($P < 0.01$) – Fig. 3A.

Fig. 3B displays the 20 most enriched GO terms, which include cell signaling and cell proliferation. Through KEGG pathway enrichment analysis, 233 pathways were identified, with 127 exhibiting significant enrichment (Fig. 4A). Fig. 4B highlights the top 20 KEGG-enriched pathways, the TNF signaling pathway related to cancer being the highlight of our analysis.

Experimental verification of predicted pathway

To validate the hypothesized molecular mechanism of Kangai injection in gastric cancer, we focused on the predicted TNF receptor (TNFR)2/PI3K/AKT/nuclear factor kappa B (NF-κB) pathway for its role in cell survival. We assessed the effects of Kangai injection on the expression levels of related genes in human gastric HGC-27 cells using RT-qPCR, while in NCI-N87

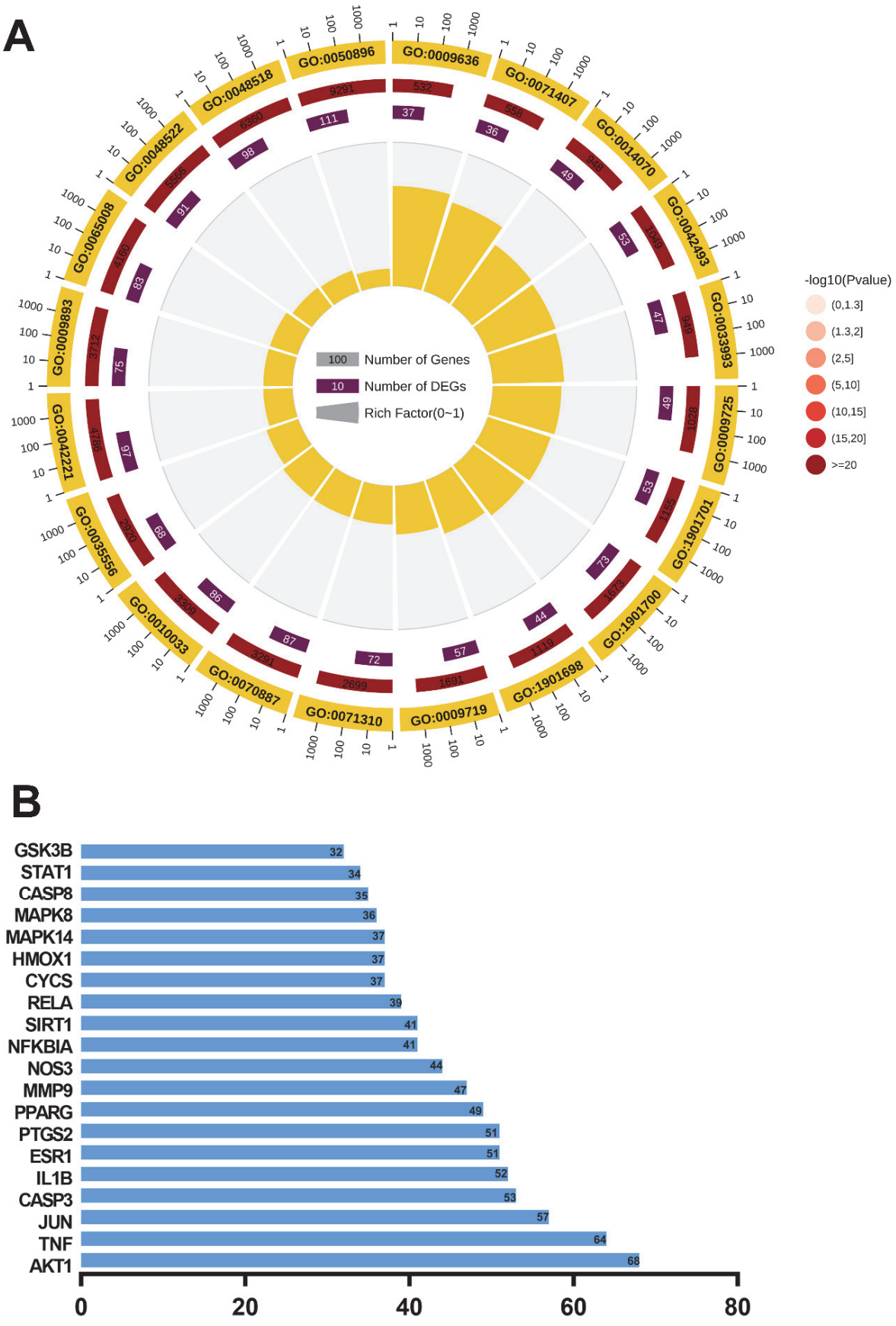

Fig. 3. The results of Gene Ontology terms. (**A**) Gene ontology analysis of the overlapping genes associated with Kangai injection and gastric cancer. (**B**) Bar plot for the first 20 target proteins based on degree value.

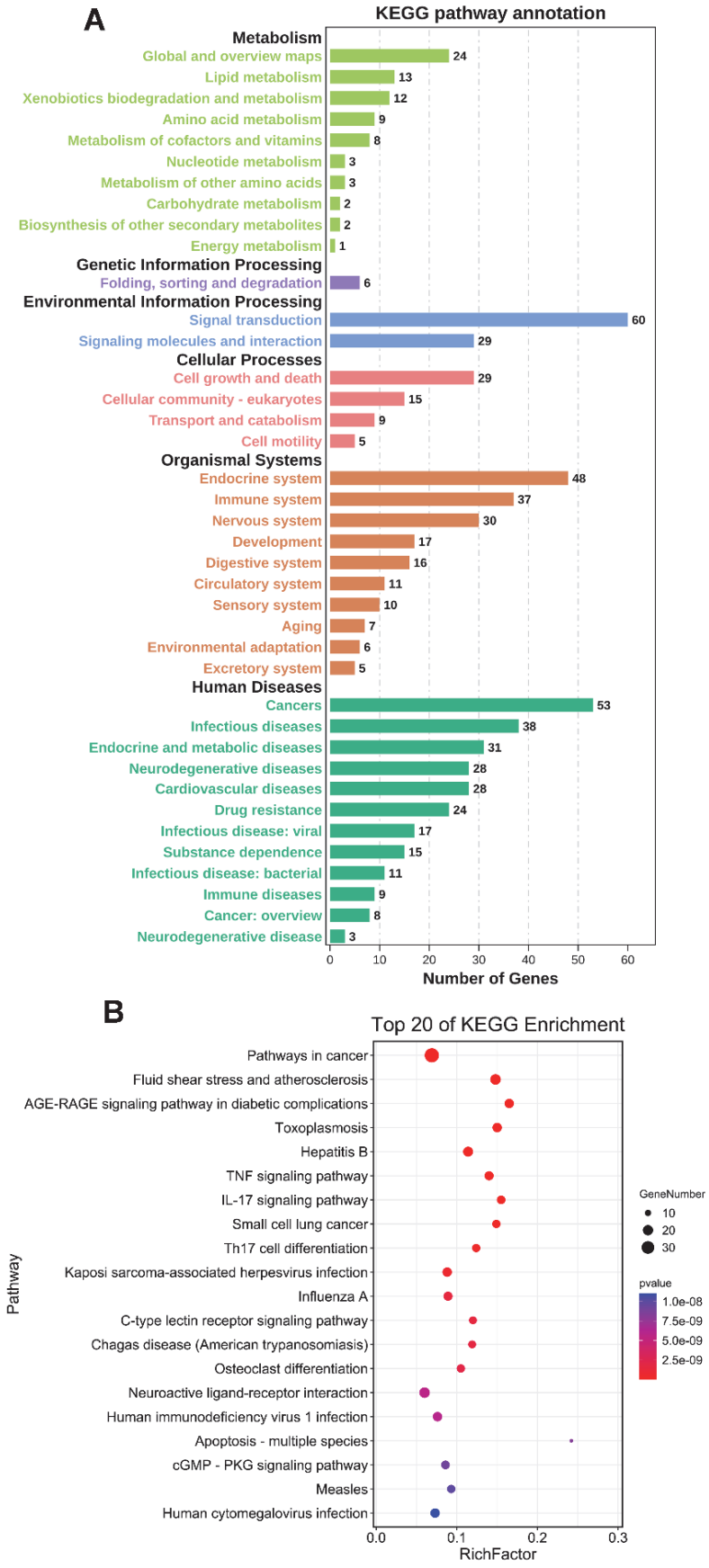

Fig. 4. The results of KEGG pathway enrichment analysis. (**A**) The annotation of KEGG pathways enriched by the target genes related to gastric cancer and by Kangai injection. (**B**) The top 20 KEGG pathways enriched by hub target genes.

cells the related proteins were analyzed by Western blot. As illustrated in Fig. 5A, Kangai injection led to the downregulation of *TNFR2*, TNFR Associated Factor 2 (*TRAF2*), *PI3K, AKT*, and inhibitor of nuclear factor kappa B (*IκBα*) at the mRNA level ($P < 0.01$). Furthermore, Kangai injection reduced the protein levels of TNFR2, TRAF2, p-PI3K, p-AKT, and p-IκBα. The PI3K activator 740 Y-P partially restored the levels of p-AKT and p-IκBα ($P < 0.05$) – Fig. 5B. MTT assays revealed that Kangai injection significantly inhibited gastric cancer cell growth, while 740 Y-P or TNFR2 overexpression partially restored their proliferative capacity ($P < 0.05$) (Figs 5C and 5D). These findings suggest that Kangai injection may suppress gastric cancer progression by inhibiting cell proliferation at least partly via the TNFR2/PI3K/AKT/NF-κB pathway.

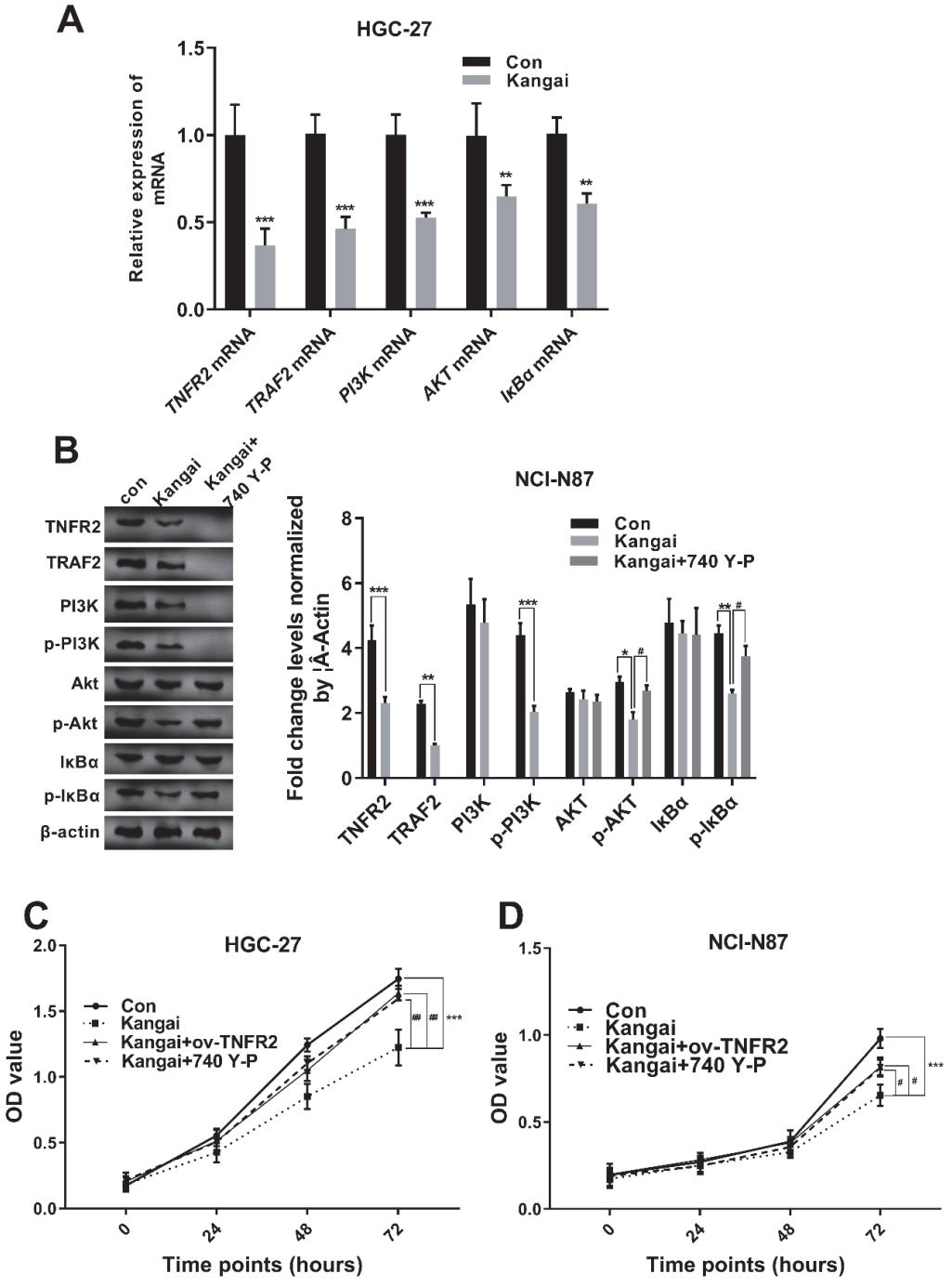

Fig. 5. Kangai injection may suppress gastric cancer cell proliferation via TNFR2/PI3K/AKT/NF-κB pathway. (**A**) RT-qPCR determined the mRNA levels in HGC-27 cells. Significance was obtained by t-test. ** $P < 0.01$, *** $P < 0.001$. (**B**) Western blotting determined the protein levels in NCI-N87 cells. Significance was obtained by *t*-test or analysis of variance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Kangai-treated group vs control group). # $P < 0.05$ (Kangai+740 Y-P-treated group vs Kangai-treated group). (**C, D**) MTT assay monitored the proliferative ability of HGC-27 and NCI-N87 cells. Significance was obtained by analysis of variance. *** $P < 0.001$ (Kangai-treated group vs control group). # $P < 0.05$, ## $P < 0.01$ (Kangai+740 Y-P-treated group vs Kangai-treated group).

Discussion

Despite advances in medical therapies, gastric cancer remains one of the most challenging malignant tumors in humans (Thrift and El-Serag, 2020). Alarmingly, cancer cells are developing resistance to various anti-tumor drugs, underscoring the need for novel antitumor agents (Chen et al., 2020). Moreover, while synthetic anti-tumor drugs can be effective, they often exhibit broad cytotoxic effects, leading to adverse reactions (Niu et al., 2020). Consequently, there is a growing interest in plant-derived phytochemicals for cancer therapy (Efferth, 2017). Kangai injection, a traditional Chinese medicine formula derived from two herbs and one phytochemical constituent, has demonstrated anti-tumor efficacy in several clinical trials across various cancers, including gastric cancer (Wang et al., 2020; Yang et al., 2021).

Considering the complex nature of Chinese traditional formulas, comprising multiple components and targeting various substrates, the emerging field of network pharmacology offers a promising approach to elucidate the underlying mechanisms. It provides an in-depth analysis of the interactions between drugs and their target protein mapping out extensive networks of these relationships.

In this study, we employed network pharmacology to investigate the potential mechanism of Kangai injection in gastric cancer and subsequently validated one of the predicted cancer-related pathways in human gastric cancer cells.

We identified the active chemical compounds in Kangai injection. Compounds such as ginsenoside, formononetin, and oxymatrine, have been previously detected in Kangai injection using liquid chromatography. For instance, Guo and Liu (2019) isolated eight compounds from Kangai injection, with matrine and ginsenosides identified as its fundamental components. Yuan (2017) also determined the presence of calycosin, ginsenoside, and formononetin in Kangai injection. Additionally, kaempferol found in both herbs used in Kangai injection, has been shown to inhibit gastric cancer both *in vitro* and *in vivo*. These previous studies validate the predictions made in this study.

Further analysis of the predicted targets revealed that 123 genes targeted by Kangai injection overlap with gastric cancer-related genes. Combining the results from the PPI network, Gene Ontology, and KEGG pathway enrichment analyses, we concluded that the TNF-related signaling pathway may be the key target of Kangai injection in gastric cancer. Data from the DAVID database suggests that Kangai injection may influence TNF, leading to a chain reaction involving TNFR2, TRAF2, PI3K, AKT, and IκBα.

Among these molecules, TNF and TNFR2 have been reported as being upregulated in gastric cancers and are related to unfavorable outcomes (Qu et al., 2022; Rossi et al., 2019). TNFR2 promotes the phosphorylation of AKT via TNF-related apoptosis-inducing ligand, thereby activating PI3K/AKT pathway (Wang et al., 2010). The following phosphorylation of IκBα activates the NF-κB signaling (Zhu and Wen, 2018).

Among the active compounds in Kangai injection, kaempferol, and ginsenoside rh2 both target TNF. Kaempferol can modulate TNFR2 expression, counteracting TNF signaling (Gupta et al., 2014; Ling et al., 2021). Ginsenoside rh2 reduces the protein expression levels of TRAF2 and regulates the PI3K/Akt and NF-κB signaling pathways (Li et al., 2020; Ren et al., 2018). Therefore, the TNFR2/PI3K/AKT/NF-κB pathway might be one of the key pathways modulated by Kangai injection to inhibit gastric cancer. Our *in vitro* tests supported these findings. However, this study only explored the preliminary mechanism of Kangai injection employing an *in silico* network pharmacology approach. Future *in vivo* validation for each constituent molecule and every related pathway is essential for understanding the precise mechanism of Kangai injection against gastric cancer.

Conclusion

This study used network pharmacology to analyze the multi-components of Kangai injection, as well as its multiple targets and related pathways against gastric cancer. The PPI network and Gene Ontology enrichment analyses indicated the central role of TNF. KEGG pathway enrichment analysis, coupled with *in vitro* experiments, provided evidence that Kangai injection may suppress gastric cancer at least partly through the TNFR2/PI3K/AKT/NF-κB pathway.

Funding
This research was funded by the 2021 ShangRao Municipal Science and Technology Program in the Field of Social Development (Grant number: 2021CZDX07).

Ethical aspects and conflict of interest
The authors report there are no competing interest to declare.

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