A novel multi-scale bioinformatics strategy for identifying the molecular mechanism by insighting into ceRNA network integrating WGCNA and meta-analysis: compound kushen injection for treating gastric carcinoma as a proof

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Keywords: Compound kushen injection, gastric cancer, ceRNA network, molecular mechanism

DOI: https://doi.org/10.21203/rs.3.rs-67746/v1

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Abstract

Background

Gastric carcinoma (GC) is a severe digestive system tumor with high morbidity and mortality and poor prognosis, of which the novel treatments are urgently needed. Compound kushen injection (CKI), a classic Chinese medicine injection, has been widely used to treat a variety of tumors in clinical for decades. In recent years, a growing number of studies have confirmed that CKI has a favorable therapeutic effect on GC, but there are few reports on the potential molecular mechanism of action.

Methods

Here, using network pharmacology as the core concept, we identified the ceRNA network and key targets of CKI in the treatment of GC. In order to further explore the impact of key targets, we conducted a meta-analysis of them and compared the expression differences between GC tissues and normal tissues. Functional analysis was utilized to understand the biological regulation pathways involved in key genes. Moreover, we further detected the significance of key genes for the prognosis of GC through survival analysis and immune infiltration analysis. Finally, molecular docking simulation was adopted so as to verify the combination of CKI components and key targets.

Results

Analysis of the ceRNA network of CKI on GC illustrated that the potential molecular mechanism of CKI was possible to regulate PI3K-AKT and Toll-like receptor signaling pathways by intervening hub genes including AKR1B1, MMP2 and PTGERR3.

Conclusions

In conclusion, this study not only partially highlighted the molecular mechanism of CKI in GC therapy but also provided a novel strategy for exploring the effective mechanisms of traditional Chinese medicine formulations.

Background

Gastric carcinoma (GC) is a malignant tumor of the digestive system that seriously threatens human health [1]. According to the statistics of the International Agency for Research on Cancer, there were about 1 million new cases of stomach cancer in the world in 2018, and about 783,000 deaths due to stomach cancer, ranking fifth in the incidence of malignant tumors and third in mortality [2]. The morbidity and mortality of GC have declined sharply in some Western countries in the past few decades, while it is still relatively high in East Asia and imposes a substantial medical burden [3, 4]. Among the factors that increase the risk of human GC, Helicobacter pylori gastric infection plays a particularly important role, and 75% of GC cases worldwide are caused by Helicobacter pylori infection [5]. Although GC is highly treatable in the early stages, the median survival time of advanced GC is only 9–10 months [6]. Unsatisfactorily, the global 5-year survival rate of patients is still less than 30% [7]. Combining different forms and different drugs of chemotherapy and radiotherapy and surgery are common methods of treating GC [8]. However, because of the internal
metastasis and changes of the tumor, the heterogeneity of different patients and the side effects of radiotherapy and chemotherapy, the options of patients in clinical practice are very limited [1].

Traditional Chinese medicine (TCM) as a complementary therapy has gradually entered the world's horizon in recent years. Compound kushen injection (CKI) which is also named compound Kushen injection is composed of Kushen (Radix Sophorae flavescentis) and Baituling (Rhizoma Smilacisglabrae) [9]. CKI has been adopted clinically to treat various types of solid tumors, including GC, liver cancer, lung cancer, breast cancer, and other cancer types for decade years [10–12]. It is worth noting that CKI can also relieve cancer pain, regulate immunity and improve conventional chemotherapy to reduce tumor efficacy and reduce chemotherapy toxicity [13, 14]. The anti-tumor effect of CKI has been confirmed whereas its underlying molecular mechanism remain poorly understood.

Molecular studies have yielded a vast quantity of new information for potential mechanisms for cancer treatment exploitation. Microarray and high-throughput sequencing technologies provide a reliable guarantee for deciphering key genetic or epigenetic changes in carcinogenesis and discovering potential biomarkers for cancer diagnosis, treatment, and prognosis [15]. MicroRNA (miRNA) and long noncoding RNA (lncRNA) are the two most common subtypes of noncoding RNA (ncRNA). Their abnormality leads to the inability of mRNA to be transcribed normally, and may contribute to unrestricted growth and invasion of cancer cells [16, 17]. At present, studies have confirmed that the lncRNA-miRNA-mRNA network plays an important role in the occurrence and development of cancer, which may have huge clinical prospects for identifying potential biomarkers and therapeutic targets of various tumors [18, 19].

Thus, in this study, we firstly analyzed the microarray dataset in the Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA) to find miRNAs that are differentially expressed in GC compared to normal tissues. Secondly, weighted gene co-expression network analysis (WGCNA) was applied and merged with differentially expressed miRNAs (DEMs) predicted targets to systematically identify genes associated with GC progression. Afterwards, we here undertake a systematic study of the molecular mechanism of CKI in the treatment of GC using a network pharmacology analytical model. Drawing on the above research, we conducted a meta-analysis of key targets to verify their expression changes in GC and conduct immune infiltration to explore their prognostic impact on GC patients. At last, in order to better analyze and predict the molecular mechanism of CKI on GC, enrichment analysis and molecular docking were exploited to discover the involved pathways and the binding of CKI components to key targets. Figure 1 depicts a flowchart of the technical strategy used in this study.

Materials And Methods

Construction of CKI ingredient prediction target network

a. Candidate compound screening for CKI

In order to obtain the active ingredients of CKI, we have conducted a literature research [10, 20]. Sixteen active ingredients in CKI were selected for the next study, and the three-dimensional structures of active ingredients were derived through the Pubchem database [21].
b. Prediction of CKI putative target

For a more profound and comprehensive study, we input the 3D structure of the chemical composition into the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) [22], Search Tool for Interactions of Chemicals (STITCH) [23], SuperPred [24], SwissTargetPrediction [25] for target prediction. Furthermore, the predicted multiple target information of the compounds and the obtained information were introduced into Cytoscape 3.6.1 [26] (http://www.cytoscape.org/) to obtain an intermolecular interaction network and carry out complex network analyses.

Differentially expressed miRNA analysis and target prediction of GC

a. Differential Expression of miRNAs in GC

Microarray data on gene expression GSE23739 was downloaded from the GEO database. A total of 80 samples were obtained, including 40 primary tumors and 40 normal samples. After the Raw data has undergone background correction and standardization, the Limma [27] R package was applied to analyze the difference between cancer and normal tissues. The miRNA-seq data in TCGA contains 446 tumor samples and 45 normal samples. In order to verify and get DEMs, the edgeR [28] package was used to analyze the difference between groups.

b. DEMs target genes prediction

MiRWalk2.0 [29] is a comprehensive archive that fully integrates the interactions of multiple existing miRNA target prediction databases and provides predictable and experimentally verified miRNA target prediction. On the one hand, the interactions between miRNAs-genes were speculated by 12 servers and only those genes projected by more than six of the servers were identified as target genes. On the other hand, 5 servers with miRWalk, miRanda, PITA, RNAhybrid and Targetscan were utilized to prognosticate miRNA-IncRNA targets.

Weighted gene co-expression network analysis for GC mRNA

a. Data collection and preprocessing

The TCGA-STAD RNA-seq data includes 407 samples of its HTSeq – Counts data and related clinical information, which was downloaded in February 2020. After removing samples containing incomplete analytical data and/or other malignancies, 375 samples were retained. Since some genes without significant changes in expression between samples we chose the top 5000 genes that are most important for differential expression for the next WGCNA analysis.

b. Weighted gene co-expression network analysis and module preservation
The gene co-expression networks were constructed by the WGCNA package [30]. The similarity between gene expression profiles was used to construct a similarity matrix based on pairwise Pearson correlation coefficient matrices. In order to improve co-expression similarity and achieve a scale-free topology, an appropriate soft threshold power $\beta$ was selected by using the integration function (pickSoftThreshold) in the WGCNA software package [31]. Besides, we have reconstructed the topological overlap matrix by the calculated the Topological Overlap Measure (TOM) which is a robust measure of network interconnectedness [32, 33]. At last, the Dynamic Tree-Cut algorithm method was adopted to identify the module of gene co-expression with the maxBlockSize of 6000, minModuleSize of 30 and mergeCutHeight of 0.2.

**c. Identification of Clinical Significant Modules**

Module eigengene (ME) means the first principal component of each gene module and the expression of ME is considered as a representative of all genes in one module. The Module Membership (MM) is the correlation between the ME and the gene expression profile. Gene Significance (GS) is the absolute value of the correlation between a specific gene and a clinical trait. According to ME, GS, MM, we can associate modules with clinical traits, not only to calculate the correlation between ME and clinical traits, but also to analyze clinically vital modules [30].

**Prediction of ceRNA network of YS intervention in GC**

In order to systematically describe GC-associated underlying molecular mechanism, a competing endogenous RNA (ceRNA) network was conducted by merging the prediction correlation of DEMs and key modules in WGCNA. The CKI active component predicted target network was combined with the ceRNA network of GC for CKI intervention in GC ceRNA network prediction, while the overlapping proteins in the two networks are likely to be the potential key gene for the treatment of GC by CKI active ingredients. A potential ceRNA network for CKI treatment of gastric cancer was constructed by Cytoscape, and the potential targets for CKI treatment of gastric cancer were systematically analyzed.

**Functional enrichment analysis**

In order to analyze the enrichment of key proteins, we first used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 10.5 (https://string-db.org/) database to construct a protein-protein interaction (PPI) network [34] for key proteins. We performed the Gene Ontology (GO) Functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the predicted key targets of the CKI compounds applied in GC therapy in order to identify their biological functions. Besides, R package clusterProfiler was used to perform GO and KEGG functional enrichment analysis. Particularly, the function and pathway enrichment analyses of the validated target genes of miRNAs, were used by the DIANA tool which is based on the cooperation of the previously-mentioned database (TarBase v7.0) and the mirPathv3.0 (a miRNA pathway analysis web server deciphering miRNA function with experimental support) [35, 36].

**Comprehensive meta-analysis of the hub gene**

**a. Data collection**
A microarray search of hub genes was conducted in the GEO database with the following terms: ("stomach neoplasms"[MeSH Terms] OR gastric cancer[All Fields]) AND "Homo sapiens"[porgn] AND ("gse"[Filter] AND "Expression profiling by array"[Filter]) and the latest searching time was April 5, 2020. The criteria for inclusion were as follows: (1) patients diagnosed with stomach cancer were investigated; (2) cancerous and noncancerous samples were involved; (3) datasets samples were no less than 20. Additionally, the following conditions caused the exclusion of a study: (1) lack of original data; (2) the patients with stomach cancer were accompanied by other tumors (3) the interventions included surgery, radiotherapy or other cancer treatment.

b. Statistical analysis and comprehensive meta-analysis

The expression profiling information of the datasets were exploited to calculate mean (M) and standard deviation (SD) of each hub gene in control experimental group. Thereafter, the meta package of R software was brought into play the standardized meta-difference (SMD) and 95% confidential interval (CI) analysis. Furthermore, in order to determine a reasonable choice of random effects and fixed effects models and heterogeneity evaluation, the chi-squared test of Q and the $I^2$ statistic were calculated.

Survival analysis of hub genes

The correlation between hub gene expression and overall survival was assessed using the Kaplan-Meier estimation method, based on the “survival” package in R. A significant difference of survival curves was assessed by a log-rank test. $P$ value less than 0.05 was considered as statistically significant.

Immune infiltrates analysis

TIMER (https://cistrome.shinyapps.io/timer/) is a database that can comprehensively study the molecular characterization of tumor-immunity interactions. Not only can the association between immune infiltrates and a variety of factors be explored interactively but the dynamic analysis and visualization of these associations can be done with a TIMER. In this study, we evaluated the hub gene expression in GC and its correlation with the abundance of tumor-infiltrating immune cells, via gene modules [37].

Molecular docking simulation

Molecular docking can reflect the binding energetics of drug molecules to protein receptors by calculating the binding affinity between ligands and receptors and the corresponding intermolecular interactions [38, 39]. The crystal structure of the key gene was downloaded from the Protein Data Bank (PDB) (https://www.rcsb.org/) database. The 3D protein crystal structure needed to be determined by X-ray crystallography and the crystal resolution was less than 3 Å. The protein receptor and ligand files were pre-processed by AutoDock Tools and then Autodock venue was used for molecular docking [40, 41]. In addition, Pymol and Ligplot were used to visualize the results so as to show the intermolecular interaction and docking more clearly [42, 43].

Results

CKI-predicted target network
Basic information on the 16 active ingredients in CKI is shown in Table 1. The active compound-predicted target network (Figure 2) consists of 301 nodes (16 compound points and 285 gene points) that constitute 635 active compound-predicted target linkages.

| PubChem CID | COMPOUND | STRUCTURE | PubChem CID | COMPOUND | STRUCTURE |
|-------------|----------|-----------|-------------|----------|-----------|
| 15385684    | 9t-hydroxymatrine |          | 87752       | lamprolobine |          |
| 190         | adenine  |          | 226371      | liroside   |          |
| 621307      | bapifoline |         | 9576780     | macrozamin |          |
| 5271984     | isomatrine |          | 91466       | matrine    |          |
| 115269      | sophocarpine |        | 670971      | N-methylcytisine |  |
| 12442899    | sophoranol |         | 24864132    | oxymatrine |          |
| 165549      | sophoridine |        | 24721085    | oxysophocarpine |  |
| 442827      | trifolirhizin |       | 6710641     | piscidic acid |      |

**Screening of differential miRNA in GC**

In this study, GSE23739 and TCGA was adopted to analysis the DEMs in GC. $|\log2FC| \geq 1$, $P$ value $< 0.05$ and adjust $P$ value $< 0.05$ were considered statistically significant for the DEMs. For GSE23739, a total of 13 up-regulated gene and 15 down-regulated genes were found. And there are 107 up-regulated genes and 56 down-regulated genes in the TCGA analysis (Figure 3). Overlapping DEMs (hsa-miR-20a, hsa-miR-30a, hsa-miR-21, hsa-miR-145) between the GSE23739 and TCGA analysis were retained for further study. From miRWALK2.0, 9431 mRNA and 31879 ncRNA targeted by DEMs (hsa-miR-20a-3p, hsa-miR-20a-5p, hsa-miR-30a-3p, hsa-miR-30a-5p, hsa-miR-21-3p, hsa-miR-21-5p, hsa-miR-145-3p, hsa-miR-145-5p) in GC predicted by more than half of total algorithms were obtained (Supplemental Table S1 to 2).
Construction and Screening of WGCNA Key Modules

After normalization, no outlier samples were eliminated in present study. In order to build a scale-free network, the power of $\beta = 6$ (scale free $R^2 = 0.85$) was selected as the soft-thresholding parameter (Figure 4A and B). A total of 9 modules were identified via the average linkage hierarchical clustering. Clinical traits including vital status, new tumor events, cancer status, pathologic T, pathologic N, pathologic M, stage, *H pylori*, barretts esophagus were selected to calculate the correlation between the module and the Pearson test. Assessed by the Pearson test when $P < 0.05$, the module and clinical characteristics were considered statistically significant. As shown in Figure 4C, the blue, turquoise and brown modules were highly correlated with clinical traits and were identified as key modules. Figure 4D indicated the topological overlap measurement (TOM) heat map of adjacency or topological overlap. TOM plot was made up by randomly selected 400 genes. Each row and column represented a module and the genes of the module. The TOM of co-expressed RNA in key modules was high, and the internal RNA correlation was also stronger. The network building the key modules was filtered with a weight Cutoff = 0.1 between the genes. The blue module consists of 632 genes and 74485 gene linkages. The turquoise module consists of 1239 genes and 126102 gene linkages and the brown module consists of 232 genes and 11316 gene linkages. (Supplemental Table S3 to 5)

Prediction of ceRNA network of CKI intervention in GC

The intersection of the WGCNA key module network and the hub DEMs prediction target constitutes the ceRNA Network of lncRNA-miRNA-mRNA Axis in GC. Furthermore, the ceRNA network and CKI-predicted target network were merged through Cytoscape to obtain prediction of ceRNA network of CKI intervention in GC (Figure 5A). As is shown in Figure 5B, the prediction of ceRNA network of CKI intervention in GC involved 73 nodes and 203 linkages between genes. The overlapping targets (AKR1B1, TLR4, ESR1, PRKCQ, PIK3CD, CTSK, MMP2, ADRB2, PDE1C, ITGB3, PDE10A, PTGFR, AR and PTGER3) were considered to be the key genes for the CKI treatment of GC.

Go and Kegg pathway enrichment analysis

A total of 14 putative targets were uploaded to the STRING database to identify the functional partnerships and interactions between them. The key genes and their interacting proteins form the PPI network for functional enrichment analysis (Supplemental Fig.1). For further interpretation of the function of the key gene, KEGG and GO annotations were performed in R software. A total of 127 GO entries were identified, including 93 biological process (BP), 25 molecular function (MF), and 9 cellular component (CC) (FDR < 0.01 and $P < 0.01$). The top ten GO terms were tissue homeostasis, anatomical structure homeostasis, toll-like receptor signaling pathway, bone resorption, intracellular receptor signaling pathway, multicellular organismal homeostasis, integrin complex, protein complex involved in cell adhesion, tissue remodeling, response to ketone (Figure6A-C). The KEGG results demonstrated that 37 entries satisfy FDR < 0.05 and $P < 0.05$ (Figure 6D). These targets were significantly enriched in many pathways related to cancer and signaling pathways, such as the PI3K-Akt signaling pathway. In addition, there was also Toll-like receptor signaling pathway related to immunity significantly enriched (Figure 7).

We also conducted a modular analysis of lncRNA-miRNA-mRNA Axis intervened by CKI in Cytoscape by Mcode. A total of key modules were analyzed including CTSK, ITGB3, PTGER3, hsa-miR-20a-5p and hsa-miR-
30a-5p five targets (Figure 8A). To gain insights into the pharmacological mechanisms of CKI on GC, we performed KEGG analysis for two key miRNAs. The results illustrated that the validated targets of hsa-miR-20a-5p and hsa-miR-30a-5p were both associated with the pathways closely related to the occurrence and development of cancer, such as Pathways in cancer, Hippo signaling pathway and p53 signaling pathway (Figure 8B).

Results of meta-analysis

A total of 8 microarrays from the GEO database met the entry criteria. The features of the included GEO datasets are depicted in Table 2. The expression data from the tumor and control groups were collected on the basis of the GEO database. A meta-analysis was conducted on the basis of expression data of the 8 included microarrays. The results (Figure 9) demonstrated that 7 of the key genes were remarkable abnormal regulation in the stomach cancer groups. Given the apparent heterogeneity, a random effects model was applied. On the one hand, ADRB2 (SMD = −1.46; 95% CI −2.02, −0.91; p <0.01), PDE1C (SMD = −0.75; 95% CI −1.11, −0.39; p <0.01) and PTGER3 (SMD = −0.58; 95% CI −1.08, −0.07; p <0.01) were down-regulation in the cancer groups which might be the tumor suppressor gene. On the other hand, AKR1B1 (SMD = 0.3; 95% CI 0.01; 0.60; p <0.01), CTSK (SMD = 1.52; 95% CI 0.98; 2.06; p <0.01), MMP2 (SMD = 1.02; 95% CI 0.51; 1.53; p <0.01), TLR4 (SMD = 0.85; 95% CI 0.34; 1.37; p <0.01) were up-regulation in the cancer groups which might be the tumor proto-oncogene. A sensitivity analysis was later conducted to explore whether a particular microarray played a vital role in significant heterogeneity. No study was found to have played a crucial role in any of the enrolled studies. A funnel plot was generated to estimate publication bias (Figure 10). The points in the funnel were distributed asymmetrically on both sides of the midline, indicating that the bias was mainly related to publication bias, but there might also be other reasons such as the lack of included literature (Figure 11).

Table 2. Features of the enrolled Gene Expression Omnibus datasets

| Accession | GPL   | Year | GC-count | Control-count | Source |
|-----------|-------|------|----------|---------------|--------|
| GSE2685   | GPL80 | 2005 | 22       | 8             | tissue |
| GSE19826  | GPL570| 2010 | 12       | 12            | tissue |
| GSE27342  | GPL5175 | 2011 | 80       | 80            | tissue |
| GSE33335  | GPL5175 | 2012 | 25       | 25            | tissue |
| GSE54129  | GPL570 | 2017 | 111      | 21            | tissue |
| GSE63089  | GPL5175 | 2014 | 45       | 45            | tissue |
| GSE79973  | GPL5175 | 2016 | 10       | 10            | tissue |
| GSE29998  | GPL6947 | 2012 | 50       | 49            | tissue |
A Kaplan–Meier curve was later used to identify the effects of the expression of hub genes on survival time. As is shown in Figure 12, AKR1B1 ($p=0.000988$), AR ($p=0.0102$), ITGB3 ($p=0.0389$), MMP2 ($p=0.0465$), PTGER3 ($p=0.0449$) and PTGFR ($p=0.0439$) with the $p$ values were all less than 0.05, thus indicated that these genes may be the key targets affecting the survival of GC patients.

**Immunohistochemical analysis of hub genes**

Through the above analysis, it was found that AKR1B1, MMP2 and PTGER3 were statistically significant in GEO chip meta-analysis and prognostic survival analysis. Therefore, we considered these three genes as the hub genes of CKI in the treatment of GC. Immunohistochemical (IHC) images of human stomach tissue samples stained with antibody were also obtained from the human protein atlas (http://www.proteinatlas.org/) [44]. Furthermore, the IHC profiler Plugin was utilized to automatically score the staining of the sample through the spectral deconvolution method [45]. According to the analysis results, AKR1B1 and MMP2 were negative in normal gastric tissue and low positive in tumor tissue, whereas PTGER3 was low positive in normal tissue and negative in tumor tissue (Figure 13). These results approved our finding.

**Results of immunoinfiltration analysis**

Analysis using TIMER showed that hub genes was negatively associated with purity, and ADRB2 (cor=-0.275) was most negatively correlated with tumor purity. In addition, the key genes were strongly correlated with macrophages and dendritic cells. Wherein PTGER3 correlation of macrophages (cor=0.637) and CTSK (cor=0.624) for the relevance of dendritic cells was the strongest correlation (Table 3, Figure 14A-C). Univariate Cox survival analysis showed that among the six types of immune cells, only macrophages were associated with the survival of GC patients, which was an indicator of the survival of GC patients (Figure 14D).

**Table 3. Immunoinfiltration analysis of key targets in TIMER**
### Molecular docking

Docking studies were carried out between CKI and hub genes, and the 3D protein structures of PTGFR and PDE1C were not found in the PDB database. The molecular docking results were shown in Table 4. AR, ITGB3, AKR1B1, ADRB2 and PTGER3 were top five genes of affinities predicted for the interaction between each of the five protein targets and corresponding CKI components.

As shown in Figure 15A, oxysophocarpine binds to a pocket in AR, which is comprised of Met895, Asn705, Trp741, Leu704, Gly708, Leu707, Gln711, Met745, Met749, Phe764, Val746, Met787 and Leu873. The results in Figure 15B show that interaction between AR and sophocarpine is centered on a stable hydrophobic core consisting of several nonpolar residues (Met787, Asn705, Met895, Leu704, Leu707, Gly708, Gln711, Met745, Met749, Phe764, Met780 and Leu873). The fifteen hydrophobic bonds, including Met780, Leu704, Asn705, Met895, Gly708, Leu707, Trp741, Met745, Gln711, Met749, Phe764, Val746, Leu873, Met787 and Met742 are formed in the interaction between sophoranol and AR (Figure 15C). The action modes baptifoline of and AR are shown in Figure 15D. Hydrophobic interactions with eleven residues in AR (Leu704, Trp741, Met742, Val746, Gln711, Met749, Phe764, Arg752, Leu707, Met745 and Gly708) and one hydrogen bonds (Asn705).

Moreover, the results in Figure 15E show that oxymatrine can bind to ITGB3 by forming a hydrophobic interaction with the surrounding residues Glu536, Arg515, Asn508, Phe547, Tyr571 and Lys548. Oxymatrine could form H-bonds with Tyr571 and Ser507.

| Gene         | Purity | B Cell | CD8+ T Cell | CD4+ T Cell | Macrophage | Neutrophil | Dendritic Cell |
|--------------|--------|--------|-------------|-------------|------------|------------|----------------|
| ADRB2        | -0.275 | 0.246  | 0.321       | 0.516       | 0.521      | 0.310      | 0.454          |
| AKR1B1       | -0.100 | -0.099 | 0.337       | 0.245       | 0.419      | 0.359      | 0.495          |
| AR           | -0.143 | 0.129  | 0.129       | 0.484       | 0.618      | 0.169      | 0.352          |
| CTSK         | -0.229 | -0.162 | 0.311       | 0.292       | 0.624      | 0.433      | 0.530          |
| ESR1         | -0.216 | 0.139  | 0.461       | 0.608       | 0.615      | 0.489      | 0.621          |
| ITGB3        | -0.149 | 0.134  | 0.159       | 0.500       | 0.471      | 0.252      | 0.349          |
| MMP2         | -0.182 | -0.123 | 0.183       | 0.285       | 0.513      | 0.273      | 0.361          |
| PDE10A       | -0.115 | 0.072  | 0.176       | 0.306       | 0.442      | 0.233      | 0.340          |
| PDE1C        | -0.174 | 0.303  | 0.122       | 0.527       | 0.432      | 0.116      | 0.229          |
| PIK3CD       | -0.230 | 0.065  | 0.504       | 0.566       | 0.409      | 0.518      | 0.665          |
| PRKCQ        | -0.122 | 0.031  | 0.443       | 0.317       | 0.239      | 0.356      | 0.459          |
| PTGER3       | -0.130 | 0.061  | 0.194       | 0.448       | 0.637      | 0.199      | 0.398          |
| PTGFR        | -0.192 | 0.119  | 0.280       | 0.486       | 0.573      | 0.352      | 0.445          |
| TLR4         | -0.146 | -0.121 | 0.389       | 0.340       | 0.548      | 0.537      | 0.641          |
The docking results in this study demonstrate that the receptor–ligand interaction between liriodendrin and AKR1B1 involves both hydrophobic interactions and polar interactions. As shown in Figure 15F, their interaction is centered on a stable hydrophobic core consisting of several nonpolar residues in AKR1B1 (Pro24, Gln49, Ala212, Lys21, Val47, Trp20, Cys298, Leu301, Trp219, Pro23 and Asn50). In addition, the hydroxyls within the main chains of Leu300, Ala299, Lys211, Trp20 and Ser22 form five hydrogen bond contacts with the liriodendrin, which further stabilizes the entire interaction region.

According to the analysis shown in Figure 15G, 9α-hydroxymatrine was observed to form hydrophobic interactions with eleven residues in ADRB2 (Phe290, Ser203, Ser204, Phe289, Phe193, Asn312, Asp113, Val117, Val114, Trp286 and Ser207) and one hydrogen bonds with Thr118.

The fifth genes PTGER3 bind to sophoranol with ten hydrophobic bonds, including Gln339, Ser336, Met58, Ile340, Gln103, Thr107, Val110, Met137, Phe133 and Thr106 (Figure 15H).

**Table 4. Molecular docking information**
| Protein Name | PDB ID | Test Compounds     | Affinity (kcal/mol) | Protein Name | PDB ID | Test Compounds     | Affinity (kcal/mol) |
|--------------|--------|--------------------|---------------------|--------------|--------|--------------------|---------------------|
| AKR1B1       | 4JRI   | liriodendrin       | -7.8                | ITGB3        | 6BXJ   | isomatrine         | -6.9                |
| TLR4         | 5IJD   | oxymatrine         | -7                  | ITGB3        | 6BXJ   | lamprolobine       | -6.4                |
| ESR1         | 4XI3   | piscidic acid      | -6.7                | ITGB3        | 6BXJ   | matrine            | -7.1                |
| PRKCQ        | 1XJD   | lamprolobine       | -7                  | ITGB3        | 6BXJ   | oxymatrine         | -8.2                |
| PIK3CD       | 5IS5   | adenine            | -5.2                | ITGB3        | 6BXJ   | sophoranol         | -7.1                |
| CTSK         | 2FTD   | isomatrine         | -6.2                | ITGB3        | 6BXJ   | sophoridine        | -6.8                |
| CTSK         | 2FTD   | lamprolobine       | -6.2                | PDE10A       | 4MVH   | 9α-hydroxymatrine | -6.2                |
| CTSK         | 2FTD   | matrine            | -6.6                | PDE10A       | 4MVH   | lamprolobine       | -6.4                |
| CTSK         | 2FTD   | oxymatrine         | -6.5                | PDE10A       | 4MVH   | sophoranol         | -6.5                |
| CTSK         | 2FTD   | sophoridine        | -6.4                | AR           | 4OEA   | 9α-hydroxymatrine | -7.5                |
| MMP2         | 1QIB   | adenine            | -6.3                | AR           | 4OEA   | baptifoline        | -8.2                |
| MMP2         | 1QIB   | matrine            | -7.3                | AR           | 4OEA   | oxysophocarpine    | -9.8                |
| ADRB2        | 3NY9   | 9α-hydroxymatrine  | -7.8                | AR           | 4OEA   | sophocarpine       | -9.4                |
| ADRB2        | 3NY9   | sophoranol         | -6.6                | AR           | 4OEA   | sophoranol         | -9                  |
| PDE1C        | 1LXS   | lamprolobine       | -7.8                | PTGER3       | 6AK3   | 9α-hydroxymatrine | -7.4                |
| ITGB3        | 6BXJ   | 9α-hydroxymatrine  | -7.3                | PTGER3       | 6AK3   | sophoranol         | -7.7                |

### Discussion

Gastric cancer is one of the most common cancers and its mortality rate remains high [46]. Since GC is difficult to detect in the initial stage, the delay in diagnosis always occurs in patients with gastric cancer [47]. Therefore, there is an urgent need to develop new treatments due to the crisis situation of GC with less cure and poor prognosis. CKI is a prescription approved by the Chinese Medicine Administration of China (NMPA). Besides, it has passed the standardized Good Manufacturing Process (GMP) certification and is often used clinically to treat gastric cancer [9]. After multiple systematic reviews and meta-analysis studies, it was found that CKI can not only improve the clinical efficacy of gastric cancer patients but also alleviate the adverse effects of radiotherapy and chemotherapy [48–50]. Cancer is a complex disease arising from changes in multiple biological networks [51]. Give this, integrated bioinformatics combined network pharmacology strategy were used to reveal the mechanism underlying the effects of CKI on GC. The high-throughput data analysis method was used to find miRNAs closely related to gastric cancer for target prediction, and intersects...
with the key modules of WGCNA analysis in TCGA to identify ceRNA networks closely related to GC. Taking network pharmacology as the core concept, finally obtained the key target of CKI on gastric cancer, and 14 intersection genes were identified as hub genes. In order to further explore the impact of CKI on gastric cancer, we conducted a meta-analysis of key targets to compare the differential expression of key genes in gastric cancer tissues and normal tissues. Second, we performed functional analysis to understand the biological regulation pathways involved in key genes. In addition, survival analysis and immune infiltration analysis were used to analyze the relationship between key genes and the prognosis of gastric cancer. Finally, molecular docking simulation was used to verify the binding of CKI components to key targets.

Network pharmacology can explain the impact of drugs on the disruptions of biological networks from the perspective of macro or overall regulation, and explain the treatment of diseases from the perspective of multi-component-multi-target-multi-pathway [52]. We discovered through network pharmacology that 14 intersection genes may be the key targets for CKI treatment of GC. After a meta-analysis of the GC gene expression profile chip, it was found that 7 of these genes, including, AKR1B1, CTSK, MMP2, TLR4, ADRB2, PDE1C and PTGER3 had significant differences in gastric cancer tissues. Besides, AKR1B1, MMP2 and PTGER3 were found to be meaningful in the analysis of gastric cancer survival, so the above three genes are considered to be the most significant hub genes for CKI to treat gastric cancer and improve the prognosis of GC. AKR1B1 as a common high-expressed gene in cancer, including gastric cancer, may lead to increased proliferation, metastasis and invasion of tumor cells by driving the epithelial-tomesenchymal transition (EMT) [53, 54]. Studies have shown that in pancreatic cancer cells, ADRB2 can directly interact with and upregulate AKR1B1, promote cell proliferation and inhibit apoptosis through the ERK1/2 pathway [55]. In addition, the expression of MMP2 in AKR1B1 knockdown cancer cells also decreased significantly compared with the control group [54]. MMP2 has been implicated in the development and morphogenesis of tumors [56]. It has been demonstrated that MMP-2 can regulate the degradation of the extracellular matrix (ECM), which plays an important role in cancer development [57]. Previous studies have confirmed that matrine, an important component of CKI, can downregulate the abnormal expression of MMP2 and thus inhibit the invasion and metastasis of tumor cells [58–60]. Whole-genome analysis showed that PTGER3 is abnormally low in gastric cancer. PTGER3 can inhibit the secretion of gastric parietal cells and gastric acid [61]. The lack of PTGER3 leads to abnormal secretion of gastrin and gastric acid and accelerates the occurrence of gastric cancer [62]. In addition, PTGER3 can also up-regulate the expression of related MMP2 and AR to promote the proliferation of cancer cells and the deterioration of gastric cancer [63, 64]. Although there is no research showing the direct effect of CKI on PTGER3, there have been studies that matrine and oxymatrine can inhibit gastric acid secretion and protect the stomach, so we think this may be also an important way for CKI to treat gastric cancer [65, 66].

In this study, we performed a GO enrichment and KEGG pathway analysis to clarify the multiple mechanisms of CKI against GC from a systematic level. The key genes of CKI on GC were enriched in the PI3K-AKT signaling pathway, the Toll-like receptor signaling pathway and other pathways which was indicated by the functional enrichment analysis. With frequent alterations identified in GC, the PI3K/AKT pathway is significantly involved in gastric carcinogenesis and progression [67]. The PI3K/AKT pathway can be activated by a variety of factors including hormone and ECM pathways, thereby regulating multiple fundamental cellular activities such as cell proliferation, apoptosis and metastasis [68]. As the most common
dysregulation pathway in cancer, the PI3K/AKT pathway has received increasing attention due to its potential for target therapy in many malignancies [69]. We proposed that CKI plays a therapeutic role in the treatment of GC that is mediated by PI3K-AKT pathway, and it has been experimentally confirmed earlier. Previous studies have confirmed that the active ingredients of matrine, oxymatrine and sophoridine in CKI can treat various tumors by inhibiting the PI3K-AKT signaling pathway [70–72]. Peng et al. [73] found that matrine can inhibit the proliferation and metastasis of gastric cancer cell SGC7901 through PI3K/Akt pathway. GC is a progressive process triggered by \textit{H. pylori}-induced inflammation [74]. The initial recognition of \textit{H. pylori} involves Toll-like receptors, the central molecule in the host's inflammatory response [75]. Most studies have concluded that TLR4 is the first innate immune response against \textit{H. pylori} [76]. The innate immune response of Toll-like receptor signaling pathway in gastric tumor cells against luminal microorganisms may be involved in the destruction of host defenses and cancer progression leading to DNA damage, cell proliferation and apoptosis regulation [77]. A recent study found that matrine can down-regulate the expression of TLR4 and regulate the Toll-like receptor signaling pathway to reduce gastric mucosal damage in rats [78].

From a comprehensive analysis of the above results, we also found that immunization may be an important potential influencing factor in CKI treatment of GC, so we conducted an analysis of immune invasion of key genes for gastric cancer. Analysis found that the degree of macrophage infiltration affects the prognosis of GC patients and the expression of key genes is positively correlated with the degree of macrophage infiltration. Consistent with this, sophoridine has been proved that can educate tumor-associated macrophages (TAMs) polarize to M1-TAMs and suppressed M2-TAMs polarization through TLR4/IRF3 axis. In addition, it can inhibit the migration ability of macrophages and reshape the immune microenvironment of gastric cancer [79]. Taken together, we propose that CKI can not only directly inhibit the proliferation and metastasis of gastric cancer tumor cells, but also improve the prognosis of cancer patients through immunotherapy.

In addition to key mRNAs, we also found two important miRNAs that may be involved in CKI regulation of gastric cancer through module analysis, which are hsa-miR-20a-5p and hsa-miR-30a-5p. Analysis of miRNA's KEGG pathway demonstrated that most of the target genes are enriched in Hippo signaling pathway and p53 signaling pathway, which are closely related to the proliferation and metastasis of cancer cells. Matrine plays an important role in regulating p53 signaling pathway to inhibit cell proliferation in liver cancer, lung cancer and esophageal cancer [80–82]. Besides, matrine can also promote colorectal cancer cell apoptosis via Hippo signaling pathway [83]. It is worth mentioning that the study found sophoridine can significantly activate Hippo and p53 signaling pathways and inhibit lung cancer progression and enhance the effects of the anticancer drug cisplatin against lung cancer cells [84]. However, there are few studies on the direct effect of CKI and its active ingredients on miRNA in the treatment of gastric cancer, which needs to be confirmed by experiments in vivo and in vitro.

**Conclusions**

In conclusion, this study explored the potential molecular mechanism of action of CKI in the treatment of GC. This research established a new multidisciplinary strategy. On the basis of traditional network pharmacology, through the analysis of high-throughput chip data and WGCNA, the potential molecular mechanism of CKI in the treatment of GC was initially obtained. And chip meta-analysis methods and survival analysis were used
to verify the expression and prognosis of key genes in GC. Functional enrichment analysis and immune infiltration analysis focus on the functional impact of key genes. Finally, molecular docking was employed to verify the tightness of the binding between the target and the components. By using network pharmacology combined with multiple integrated bioinformatics method framework, we systematically revealed that CKI may be involved in regulating the ceRNA network for the treatment of GC. Among them, mRNA including AKR1B1, MMP2 and PTGER3 and miRNA including hsa-miR-20a-5p and hsa-miR-30a-5p may play an important role in the therapy. Our preliminary conclusion is that CKI can be used for GC therapy by activating signaling pathways such as PI3K/Akt and Toll-like receptor signaling pathways to inhibit cancer cell proliferation and regulate immunity. Based on multidisciplinary approach, this study might provide a new perspective for the profound exploration and provide a reference for multicomponent-multitarget-multipathway clinical research.

Abbreviations

GC, gastric carcinoma; CKI, compound kushen injection; miRNA, microRNA; lncRNA, long noncoding RNA; ncRNA, noncoding RNA; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; WGCNA, weighted gene co-expression network analysis; DEMs, differentially expressed miRNAs; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; STITCH, Search Tool for Interactions of Chemicals; TOM, Topological Overlap Measure; ME, Module eigengene; MM, Module Membership; GS, Gene Significance; ceRNA, competing endogenous RNA; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Functional and Kyoto Encyclopedia of Genes and Genomes; M, mean; SD, standard deviation; SMD, standardized meta-difference; CI, confidential interval; PDB, Protein Data Bank; NMPA, Chinese Medicine Administration of China; GMP, Good Manufacturing Process; EMT, epithelial-tomesenchymal transition; ECM, extracellular matrix; TAMs, tumor-associated macrophages.

Declarations

Funding

The design of the study and the collection, analysis, and interpretation of data were supported by the Young Scientists Training Program of Beijing University of Chinese Medicine [grant number BUCM-QNLJ 2019001], the National Nature Science Foundation of China [grant no. 81673829] and Chinese Medicine Modernization Project [2017YFC1701900].

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Author contributions

WZ, JRW and RLY designed the experiment and wrote the manuscript. WZ and XKL collected and analyzed the data. WZ, SYG and SL interpreted the data and visualized the results. JYZ and SSJ substantively revised the manuscript. All Authors gave the final approval of the version to be published.

Ethics approval and consent to participate

The ethical approval was not necessary in current study because our study just gathered the data from the open source database such as GEO and TCGA, this procedure was without deal with any patients’ personal data and harm to any patient.

Competing interests

The authors declare that they have no conflicts of interest to declare.

Consent for publication

All the patients that involved in the study have given their consent to publish their individual data.

Additional File

Supplementary data: (1) Figure S1. PPI network of key genes. (2) Table S1. mRNA targets predicted by miRNA. (3) Table S2. ncRNA targets predicted by miRNA. (4) Table S3. Blue network nodes in WGCNA. (5) Table S4. Brown network nodes in WGCNA. (6) Table S5. Turquoise network nodes in WGCNA.

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**Figures**
**Figure 1**

Flow chart of novel multi-scale bioinformatics research strategy for CKI in the treatment of gastric carcinoma.
Figure 1

Flow chart of novel multi-scale bioinformatics research strategy for CKI in the treatment of gastric carcinoma.
Figure 2

Prediction target network of CKI component. Orange dots indicate the components in YSI, and green dots indicate their predicted targets.
Figure 2

Prediction target network of CKI component. Orange dots indicate the components in YSI, and green dots indicate their predicted targets.
Figure 3
Volcano and heat maps of DEMs. (A) Volcano map of GSE23739. (B) Volcano map of TCGA. (C) Heat map of GSE23739. (D) Heat map of TCGA.
Figure 3

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Figure 4

WGCNA results for TCGA-STAD. (A) Soft-thresholding power analysis. R2=0.85. (B) Clustering dendrogram. corFnc="pearson"; power=6; min. module size=30; mergeCutHeight of 0.2. (C) Module-trait relationship. Each row corresponds to a ME, and each column corresponds to a clinical trait. Each cell contains a corresponding correlation and p-value of modules with various clinical traits. (D) Network TOM heatmap plot.
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Figure 5

(A) The Venn diagram of the prediction targets of DEMs and the key modules of WGCNA and the prediction targets of CKI. (B) The prediction of ceRNA network of CKI intervention in GC. Orange dots are the components of CKI, and pink arrows indicate miRNAs that may be involved in regulation. Blue, turquoise and brown nodes indicate the key genes of CKI intervention in GC, and the different colors correspond to the module colors in WGCNA. The gray nodes represent the intersection IncRNA in DEMs prediction and WGCNA module.
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Figure 6

Enrichment analysis of the key genes. (A) GO bubble chart of function enrichment for key genes. (B) The circular map of the key genes distribution of the top ten GO pathway. (C) The clustering map of the first ten GO pathways. (D) KEGG bubble chart of function enrichment for key genes.
Figure 6

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Figure 7

Regulatory pathways mainly involved in the CKI treatment of GC.
Figure 7

Regulatory pathways mainly involved in the CKI treatment of GC.
Figure 8

(A) Module analysis of CKI intervention ceRNA. The genes in the pink box constitute the key module. (B) Enrichment analysis of key miRNAs pathways.
Figure 8

(A) Module analysis of CKI intervention ceRNA. The genes in the pink box constitute the key module. (B) Enrichment analysis of key miRNAs pathways.
**Figure 9**

Forest plot of meta-analysis of key genes. (A) ADRB2 (B) AKR1B1 (C) CTSK (D) MMP2 (E) PDE1C (F) PTGER3 (G) TLR4
Figure 9

Forest plot of meta-analysis of key genes. (A) ADRB2 (B) AKR1B1 (C) CTSK (D) MMP2 (E) PDE1C (F) PTGER3 (G) TLR4
Figure 10

Sensitivity analysis of GEO chips of meta-analysis of key genes. (A) ADRB2 (B) AKR1B1 (C) CTSK (D) MMP2 (E) PDE1C (F) PTGER3 (G) TLR4
Figure 10

Sensitivity analysis of GEO chips of meta-analysis of key genes. (A) ADRB2 (B) AKR1B1 (C) CTSK (D) MMP2 (E) PDE1C (F) PTGER3 (G) TLR4
Figure 11

A funnel plot was applied to evaluate the publication bias of GEO datasets (A) ADRB2 (B) AKR1B1 (C) CTSK (D) MMP2 (E) PDE1C (F) PTGER3 (G) TLR4
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Figure 12
Survival analysis of key genes.
Figure 13

IHC of AKR1B1, MMP2 and PTGER3 expression in GC tissues and paired normal tissues based on The Human Protein Atlas.
Figure 13

IHC of AKR1B1, MMP2 and PTGER3 expression in GC tissues and paired normal tissues based on The Human Protein Atlas.
Figure 14

Results of immunoinfiltration analysis. (A) ADRB2 (B) CTSK (C) PTGER3 (D) Survival analysis of immune cells in GC.
Figure 14

Results of immunoinfiltration analysis. (A) ADRB2 (B) CTSK (C) PTGER3 (D) Survival analysis of immune cells in GC.
Figure 15

Molecular docking of the key gene with its corresponding component. (A) AR-oxysophocarpine (B) AR-sophocarpine (C) AR-sophoranol (D) AR-baptifoline (E) ITGB3-oxymatrine (F) AKR1B1-liriodendrin (G) ADRB2-9α-hydroxymatrine (H) PTGER3-sophoranol
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Supplementary Files

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

- TableS5.TurquoiseNetworkNodesInWGCNA.txt
• TableS5.Turquoise network nodes in WGCNA.txt
• TableS4.Brown network nodes in WGCNA.txt
• TableS4.Brown network nodes in WGCNA.txt
• TableS3.Blue network nodes in WGCNA.txt
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• TableS2.ncRNA targets predicted by miRNA.xlsx
• TableS2.ncRNA targets predicted by miRNA.xlsx
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• TableS1.mRNA targets predicted by miRNA.xlsx
• FigureS1.PPI network of key genes.png
• FigureS1.PPI network of key genes.png