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Uncovering the role of traditional Chinese medicine in immune-metabolic balance of gastritis from the perspective of Cold and Hot: Jin Hong Tablets as a case study

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Abstract

Chronic gastritis (CG) is a common inflammatory disease of chronic inflammatory lesion of gastric mucosa and in the diagnosis of gastritis in traditional Chinese medicine (TCM), CG can be classified into Cold ZHENG (syndrome in TCM) and Hot ZHENG. However, the molecular features of Cold/Hot ZHENG in CG and the mechanism of Cold/Hot herbs in formulae for CG remained unclear. In this study, we collected a transcriptomics data including 35 patients of Cold/Hot ZHENG CG and 3 scRNA-seq CG samples. And 25 formulae for CG and 89 herbs recorded in these formulae were also collected. We conduct a comprehensive analysis based on the combination of transcriptomics datasets and machine learning algorithms, to discover biomarkers for Cold/Hot ZHENG CG. Then the target profiles of the collected formulae and Cold/Hot herbs were predicted to uncover the features and biomarkers of them against Cold/Hot ZHENG CG. These biomarkers suggest that Hot ZHENG CG might be characterized by over-inflammation and exuberant metabolism, and Cold ZHENG CG showed a trend of suppression in immune regulation and energy metabolism. Biomarkers and specific pathways of Hot herbs tend to regulate immune responses and energy metabolism, while those of Cold herbs are more likely to participate in anti-inflammatory effects. Finally, the findings were verified based on public transcriptomics datasets, as well as transcriptomics and ELISA detection, taking Jin Hong tablets as a case study. Biomarkers like leptin and IL-6 together with proportions of immune cells showed significant changes after the intervention. These findings might reflect the mechanism and build a bridge between macro and micro views of Cold/Hot ZHENG as well as Cold/Hot herbs.

Keywords Cold/Hot, Chronic gastritis, Traditional Chinese medicine, Network target, Jin Hong tablets

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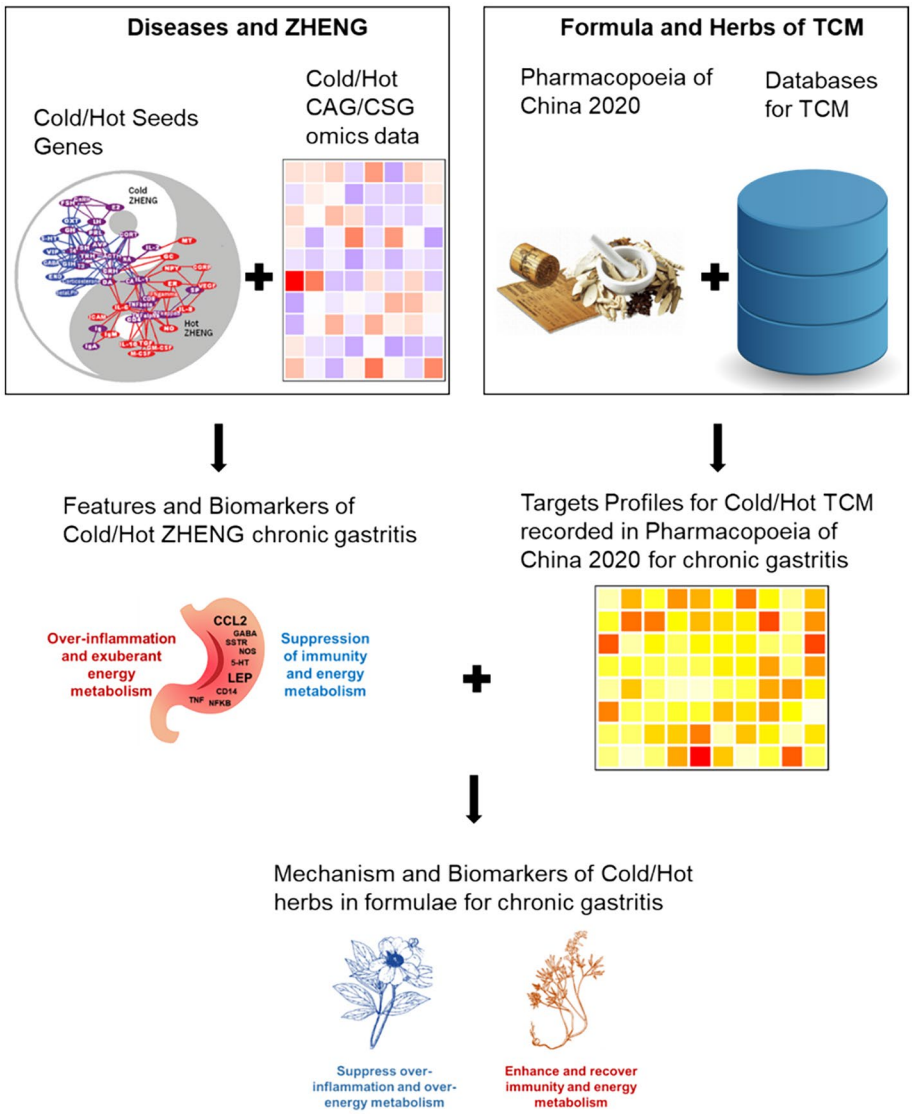
Graphical abstract



Introduction

Chronic gastritis (CG) is defined as inflammatory diseases of the gastric mucosa and is classified as chronic superficial gastritis (CSG) and chronic atrophic gastritis (CAG) based on the histopathologic patterns and endoscopic appearances of the gastric mucosa [1]. The prevalence of CG may exceed 50% worldwide [2]. The progressive deterioration of atrophic gastritis, which subsequently leads to dysfunction of the gastric mucosa, is also the highest independent risk factor for gastric cancer [3].

To date, the etiology of CG remains incompletely understood. It can be caused by a range of factors such as stress, alcohol, irrational use of nonsteroidal anti-inflammatory drugs, and *H. pylori infection* [4], leading to an imbalance between offensive acid-pepsin secretion and defensive mucosal factors such as cell shedding and mucin secretion [5]. Regarding stress risks, physiologic stress can result in dysregulation of gastric pH, which contributed to gastritis. In the stressed state, increased levels of histamine and acetylcholine result in elevated acid production, thus inducing or worsening gastritis [6]. The present therapy of gastritis is to alleviate

inflammation and associated dyspeptic symptoms, and specific treatments should be determined accordingly depending on each individual's condition. In China, traditional Chinese medicine (TCM) therapy is an important complementary treatment option for CG [7].

ZHENG, a TCM theoretical understanding of the symptomatic profiles of disease, is used to recognize and understand the non-healthy physiological states of patients from a holistic view. In TCM clinical diagnosis, there are different types of ZHENG for the same disease depending on the phenotype profiles. All diagnostic and therapeutic approaches in TCM are based on the typology of ZHENG. In the diagnosis of gastritis in TCM, the patients with CG can be classified into two main types: Cold ZHENG and Hot ZHENG. The CG associated with Cold ZHENG has characteristics of cooling of limbs, loose stool, clear abundant urine, white-greasy tongue coating. The CG associated with Hot ZHENG is characterized by red tongue, yellow-dense tongue coating, thirst, dry mouth, deep-colored urine, and dysphoria with a feverish sensation. In our previous study, we have evaluated the biological basis of CG associated with Cold/Hot ZHENG, suggesting that the metabolism-immune network imbalance has the potential to be a new perspective in the development of sub-typing and individualized treatment for CG [8]. According to the rules of TCM diagnosis and treatment, patients with Cold ZHENG should be treated by herbs with hot property (Hot herbs) and patients with Hot ZHENG should be treated by herbs with cold property (Cold herbs) for thousands of years in China. For the treatment of CG in TCM, CG associated with Cold and Hot ZHENG are treated with herbs that have hot and cold properties, respectively. However, the biological mechanisms behind Hot and Cold herbs for the treatment of Cold and Hot ZHENG remain unclear. Recent advances in TCM research are currently associated with the rapid development of concepts in network pharmacology and systems biology that provide approaches to understanding the rules of TCM diagnosis and treatment [9, 10]. The aim of this study is to investigate the mechanisms of action of Cold/Hot herbs in the treatment of CG associated with Cold/Hot ZHENG through a network pharmacology approach.

Results

Outlier of the whole study

We conducted a comprehensive analysis on gastritis and widely used TCM formulae and herbs for gastritis from the perspective of Cold/Hot ZHENG and network target in this study. First and foremost, based on seeds genes from Cold/Hot biological network [11] proposed by Li and microarray for CAG and CSG with Cold/Hot ZHENG [8], we constructed Cold/Hot ZHENG biological network for CG to discover the features and biomarkers of Cold/Hot ZHENG. On the basis of multi-omics data and machine learning algorithms, features and biomarkers of immune and metabolism were characterized and kept for following study. With the help of Cold/Hot biological network as well as the inferred immune and metabolism related characteristics, features and biomarkers of Cold/Hot ZHENG CG were acquired which were mainly related to immune regulation and metabolism.

Besides, we collected 29 formulae for GC and corresponding 132 herbs recorded in these formulae from Pharmacopoeia of China. Cold/Hot information and Meridian information of these herbs were collected from Pharmacopoeia. The distribution of Meridian information of the herbs were counted and the compounds composition of these herbs was obtained from commonly-used TCM database Herbiomap [12] and Symmap [13]. After filtering herbs without recording or without, 25 formulae, 89 Cold/Hot herbs, 19 other herbs and in total 2853 compounds were kept for further study, and the targets profiles for these herbs and formulae were characterized by our previous network-based algorithms [14, 15].

Finally, as a combination of Cold/Hot ZHENG biological network for CG and targets profiles for Cold/Hot herbs, a biological network describing the most-frequently targeted Cold/Hot genes of Cold/Hot herbs was constructed to uncover the mechanism of formulae with Cold/Hot properties and corresponding Cold/Hot herbs in formulae for CG to some extension. Based on the network and previous studies, potential biomarkers of Cold/Hot herbs in the formulae against CG were determined and their relationship with potential mechanism of Cold/Hot ZHENG were also explored to reveal the mechanism of these herbs against Cold/Hot ZHENG CG.

Molecular features of Cold/Hot ZHENG CG

In 2013, Li collected 35 patients of Cold/Hot ZHENG CG for microarray measurement, including 17 patients with Cold ZHENG (8 for chronic superficial gastritis and 9 for chronic atrophic gastritis) and 18 patients with Hot ZHENG (8 for chronic superficial gastritis and 10 for chronic atrophic gastritis). In order to find key molecules related to Cold/Hot ZHENG in CG, we collected seeds gene from previous Cold/Hot network model as background for Cold/Hot ZHENG. PLS-DA analysis successfully grouped Hot and Cold ZHENG CG for CAG patients and CSG patients, respectively (Fig. 1A). VIP (variable importance) for each gene in CAG patients or CSG patients was calculated and 26 of the seeds genes with VIP greater than 1 both occurred in CAG patients and CSG patients (Fig. 1B).

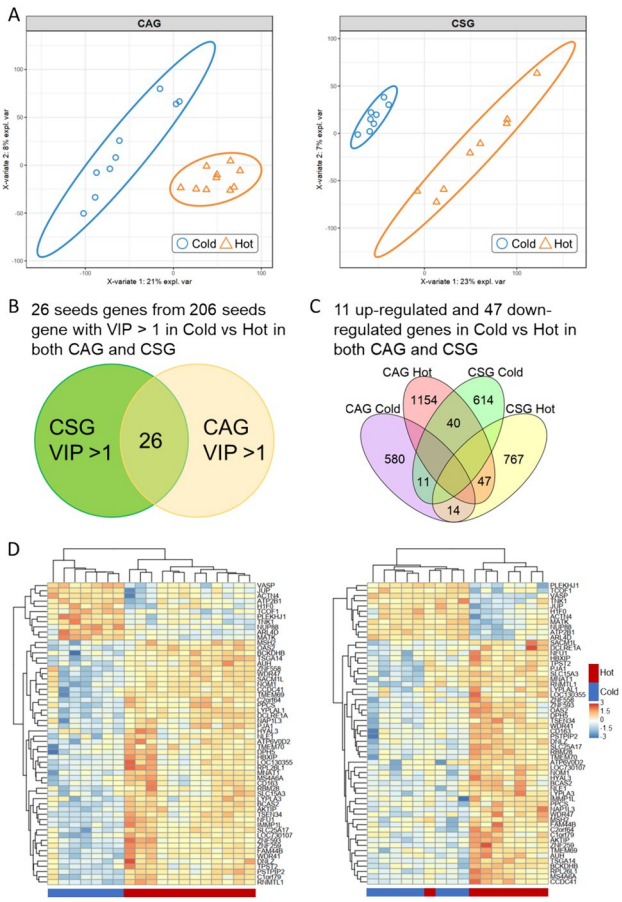


Fig. 1 Analysis for finding representative molecules between Cold/Hot ZHENG CG. **A** PLS-DA analysis for Cold/Hot ZHENG CAG (left) and CSG (right). **B** Venn plot showing genes with VIP larger than 1 in both Cold/Hot ZHENG CAG and CSG. **C** Venn plot showing the differential expression of genes in microarray of Cold/Hot ZHENG CAG and CSG. **D** Heat map of 47 genes both up-regulated in Hot ZHENG CAG and CSG and 11 genes up-regulated in Cold ZHENG CAG and CSG for CAG patients (left) and CSG patients (right)

Besides, from another perspective, DEGs (differentially expressed genes) in CAG patients and CSG patients were calculated by limma model to find significantly expressed genes between Cold/Hot ZHENG in both CAG patients and CSG patients. Among these DEGs, 112 genes were differentially expressed in both CAG patients and CSG patients (adjust P value < 0.05, BH adjustment). And 11 genes were up-regulated in both Cold ZHENG CAG patients and CSG patients, while 47 of them were up-regulated in both Hot ZHENG of two disease conditions (Fig. 1C). Combining these two analyses of different perspectives, both in statistical methods and hierarchical clustering methods, Hot ZHENG patients were distinct from Cold ZHENG patients (Fig. 1D). This result suggested that there existed gene expression patterns between these two conditions of CG which might be able to mined from our analysis and further constructed biological networks.

Immune and metabolic characteristics of Cold/Hot ZHENG CG

Based on our found molecular features of Cold/Hot ZHENG CG, we further paid attention to the biological processes or pathways enriched by these molecular features. Firstly, we performed KEGG pathway enrichment [16] and Gene Ontology (GO) enrichment [17] on 1846 DEGs (1241 genes up-regulated in Cold ZHENG and 605 genes up-regulated in Hot ZHENG) of CAG patients. It was found that pathways and biological processes related to immune and metabolism were significantly enriched (Fig. 2A). Further, we performed Gene Set Enrichment Analysis (GSEA) [18] on CAG patient and GSEA terms that significantly enriched were shown in Fig. 2. It could be found that, GSEA terms related to immune, inflammation, cytokines and chemokines (Fig. 2B–D) were activated in Hot ZHENG CAG patient (also can be defined as inhibited terms in Cold ZHENG CAG patient, NES < −1), while terms related to metabolism and secretion of peptide, hormone, steroid (Fig. 2B, E), as well as cellular junctions and adhesion were activated in Cold ZHENG CAG patient (NES > 1). These findings suggested that in Hot ZHENG CG, pathways or biological processes related to immune and inflammation might be over-developed, while in Cold ZHENG CG, the main distinguished features turned out to be activating in endocrine and energy metabolism. Apart from CG, these modules of immune regulation and metabolism had also been reported in researches about other diseases [19–21].

As inferred by our previous findings, we focused on the immune and inflammation characteristics in CG. Based on CIBERSORT algorithm [22], proportions of different immune cells were deconvoluted in Cold/Hot ZHENG CG, respectively. It could be found that some immune cells, represented by M1 macrophages, showed a significantly different proportion in Hot ZHENG CG than Cold ZHENG CG (Fig. 3A). The proportions of both M1 and M2 macrophages were significantly higher in Hot ZHENG CG than that of Cold ZHENG CG, which might confirm the findings that from the perspective of immune and inflammation regulation, the most distinguishing features between Hot ZHENG and Cold ZHENG gastritis is the over-inflammation in Hot ZHENG and the suppression of immune in Cold ZHENG.

In addition, we filter out 3 Hot ZHENG CAG samples from large scale scRNA-seq of human gastric cancer progression. In this cellular level measurement of expression of genes in these characteristic pathways and biological

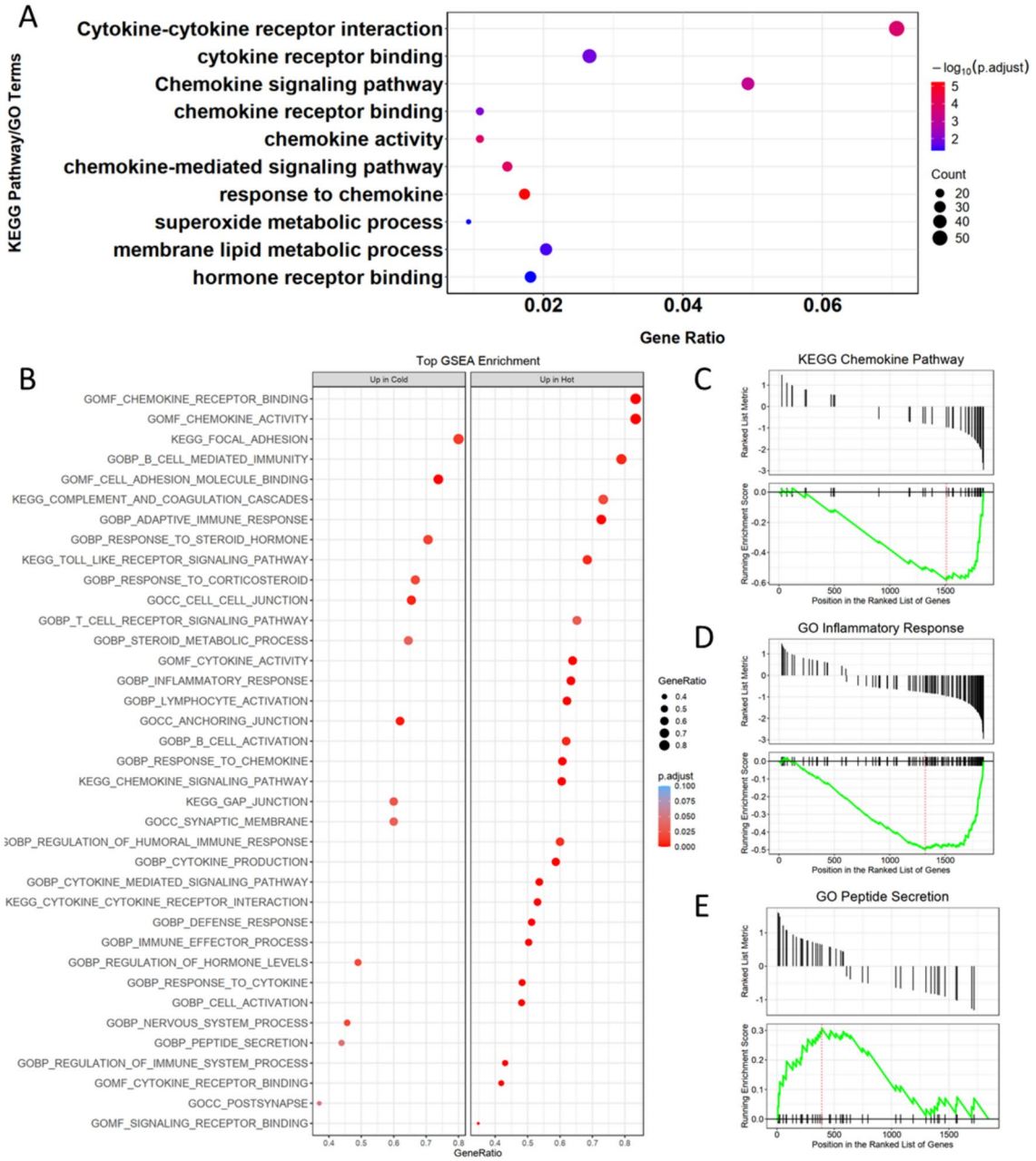


Fig. 2 Enrichment analysis for immune and metabolic characteristics of Cold/Hot ZHENG CG **A** Dot plot of KEGG and GO enrichment analysis for 1846 DEGs of CAG patients. **B** GSEA for genes and their expression of CAG patients. **C**–**E** GSEA result for Chemokine Pathway, Inflammatory Response and Peptide Secretion, respectively

processes related to immune regulation and metabolism, we focused on some key molecules in Cold/Hot ZHENG CG. It was found that biomarkers of Cold ZHENG CG which participated in these key pathways and biological processes, like HTR2B, CRH, NOS1 and LEP, hardly expressed in any cell types in Hot ZHENG CG samples. These genes were reported in previous study [8], and were found to be related to key link in Cold ZHENG, including 5-HT related gene HTR2B, corticotropin releasing hormone related genes CRH, CRHR1 and POMC, leptin related gene LEP and nitric oxide related gene NOS1. On the contrary, genes related to immune and inflammation were relatively higher expressed, especially in macrophages, which was consistent with our above findings that macrophages significantly increased in Hot ZHENG CG. These genes included CCL2, CD14,

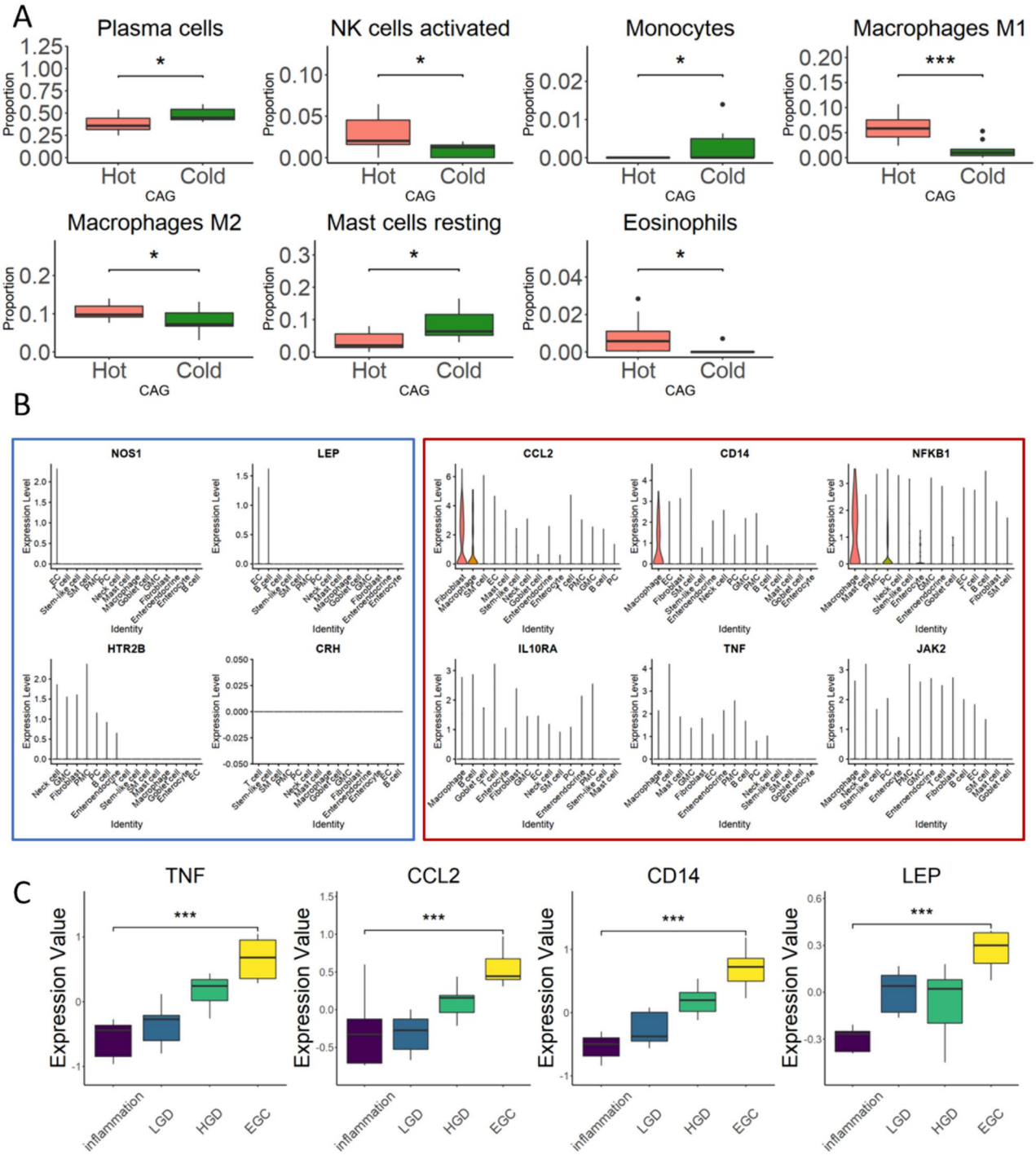


Fig. 3 Network construction of Cold/Hot ZHENG CG. **A** Inference of the proportion of immune cells significantly changed in Cold ZHENG CAG and Hot ZHENG CAG. **B** Expression of previous reported and newly found biomolecules for Cold/Hot ZHENG CAG in single-cell level of CAG patients. **C** Box plots showing the expression of biomolecules during the progression of gastric cancer

NFKB1, IL10RA, TNF and JAK2, which were related to inflammation, cytokines, chemokines and immune regulation (Fig. 3B). Based on public omics data, it was also found that some of these biomarkers showed significant changes in the progression from gastritis to dysplasia and gastric cancer (Fig. 3C), and were reported to participant in the progression of gastric cancer, such as LEP, CCL2, CD14 and TNF. The expression of these biomolecules

and their related biomolecules was found to be associated with gastric cancer progression and prognosis [23–26], which also inferred that pathways or cells related to these biomarkers may also play roles in cancer progression and prognosis. These findings inferred us that the key biomolecules in Cold/Hot ZHENG might also play important roles in other disease progressions which need further analyses and researches. These complex features in immune regulation and metabolism, as well as biomolecules like TNF, VEGF, TGFB and NFKB1, also reflect the potential risk of CG, especially CAG in inflammation-induced tumorigenesis according to our previous constructed tumorigenesis network [27], and also be reported in researches of tumorigenesis in other digestive systems diseases like chronic hepatitis and enteritis [28, 29].

Finally, based on the combination of multi-omics data and machine learning algorithms, we constructed a homogeneous biological network, composed of key seeds genes of Cold/Hot ZHENG and DEGs in both two kinds of CG (Figure S1). In this network, the interactions between every two genes were collected from STRING database. It could be found that many of the seeds genes played important roles in these network with high connections, especially immune and inflammation-related genes like CCL2, CD14, NFKB1, IL2RB, JAK2, VEGFC, TGFB3 and IL10RA, most of which showed high expression in macrophages of Hot ZHENG CG patients. Besides, some genes related to endocrine and energy metabolism including SSTR2, SSTR5, HTR1A, CRH, CRHR1 and POMC also had high degrees in this network. It is worthy to be noticed that not only the biomolecules in the network for Cold/Hot ZHENG CG, but also the genes with close functional relationship or biological relationship may play important role in the further diagnosis and mechanism uncovering of Cold/Hot ZHENG CG.

According to our enrichment result, metabolism features of CG is also of vital importance in the diagnosis of Cold/Hot ZHENG CG. Metabolism related to peptide is significantly enriched, and the interactions between peptide and protein participate in various fundamental cellular functions [30]. The pathologically elevated steroid hormones may be accompanied by leptin resistance, which weakens normal energy expenditure and thermogenesis [31]. In our previous study, we found that serum level of leptin in CAG patients associated with Cold ZHENG was significantly higher than normal subjects [8]. Therefore, the presence of pathologically elevated leptin levels in patients with cold ZHENG means that their reduced energy expenditure and thermogenesis may be due to leptin resistance. Conditional Dlx1/2-null mice showed a loss of growth hormone-releasing hormone neurons with higher somatostatin expression and lower energy expenditure [32]. A previous study also showed that somatostatin in the paraventricular nucleus of the hypothalamus could inhibit thermogenesis [33], suggesting that SSTR involves in energy expenditure. It has been reported that 5-HT could inhibit thermogenesis through Htr3 in brown adipose tissue [34]. Besides, in the median preoptic nucleus, the thermoregulatory response is initiated by stimulation of GABA neurons, suggesting that GABA plays an important role in the process of immune regulation and energy expenditure [35]. Last but not the least, the suppression of tight junction and gap junction was associated with the activation of gene networks of adaptive immunity [36]. And tight junction was reported to be related to immune suppression in COVID-19 [37].

Characteristics of formulae for CG

From the perspective of system biology, we focused on the potential effect on formulae for CG. We measured some typical pathways or biological processes for Cold/Hot ZHENG CG in our above findings in all 29 formulae recorded in Pharmacopoeia. It was found that in specific pathways or biological processes in immune regulation, inflammation and steroid dominated energy metabolism, the potential effect of different formulae differed from others in immune-related pathways and biological processes like immune cells, immune response, cytokines and chemokine. Besides, some other pathways and biological processes related to energy reverse metabolism, nitric oxide. On the contrary, steroid metabolic process, response to steroid hormone, steroid hormone mediated signaling pathway, regulation of inflammatory response and response to oxidative stress were consistently significantly enriched and might be a coincident potential mechanism of these formulae against CG (Figure S2A).

All the 25 formulae could be pasted with six labels for traditional effects, including ZI YIN, XIAO JI, SAN HAN, QING RE, HUO XUE and XING QI. According to TCM experience, these six labels were corresponded with specific effects, in which ZI YIN, XIAO JI, SAN HAN, QING RE, HUO XUE and XING QI means nourishing humors, eliminating food stagnation, dispelling Cold, removing Hot, promoting the restoration of vitality and achieving smooth air flow, respectively. XING QI was the most frequent effect for these formulae, and it was positively correlated (Wilcoxon test, P value < 0.05) with the proportion of Hot herbs in a formula (Figure S2B, C). Besides, ZI YIN had significantly positive correlation with the proportion of Cold herbs, while SAN HAN had significantly positive correlation with the proportion of Hot herbs. In addition, XIAO JI and XING QI showed a relatively positively correlation with the proportion of Hot TCM in a formula. These findings were consistent

with TCM theory that SAN HAN and XING QI are typical traditional effects of Hot and Warm herbs or formula with Warm and Hot properties, while QING RE and ZI YIN were the characteristics of Cold and Cool ones, and might uncover the material basis of these related six labels for traditional effects. Further, in the four significantly different labels for traditional effects in formulae with Cold/Hot herbs, we focused on the pathways of formulae with them to uncover the mechanism of these four traditional effects. It was found that pathways belonging to signal transduction, endocrine and immune were the most important in these four traditional effects, which might consistently support our findings that metabolism and immune regulation were the key mechanism of Cold/Hot ZHENG CG.

Herbs and depiction of their target profiles in formulae against CG

Another main finding of this study falls in the various mechanism of actions of Cold/Hot herbs in traditional formulae for CG. We collected 29 traditional formulae for CG and their corresponding herbs from Pharmacopoeia. These 29 formulae totally included 242 herbs (132 unique herbs). In annotating the compound composition for these herbs from two large databases Symmap and Herbiomap, 108 of these 132 herbs were successfully matched with the compounds as well as Cold/Hot information and meridian information. In total, 2853 unique compounds were found in these herbs and annotated with PubChem CID [38] for further targets prediction.

Target profiles of these compounds were calculated by our previous network-based algorithm DrugCIPHER-SC [14] and top 100 druggable targets of the profiles were chosen as the targets for further analysis. In order to measure the holistic targets of formulae and herbs, a previous statistical strategy [15] was performed and targets with occurrence significance less than 0.05 (BH adjustment) were chosen.

Cold/Hot information is one of the most important information in TCM herbs. According to Pharmacopoeia, the Hot and Cold properties have been divided into seven levels: Cold, light Cold, Cool, Ping (a kind of state which has no tendency to Cold or Hot), light Warm, Warm and Hot (including great Hot). The first three levels belong to Cold category and the last three levels belong to Hot category. Herbs belonging to Warm held the largest population with the amount of 45 and those of Cold held the second largest population of 31, while herbs belonging to Hot, light Warm and Cool held the least population.

Apart from Cold/Hot information, meridian was also of vital importance for herbs, for the reason that it might figure out the place where herbs took effect. Herbs in traditional formulae for CG mainly includes 12 kinds of meridian, including triple energizer (tri-jiao, a special TCM term), large intestine, small intestine, heart, pericardium, liver, lung, kidney, stomach, gallbladder, spleen and bladder. It was found that one herb might include more than one meridian, which showed that herbs might take effect in many tissues. It could be found that spleen was the most frequent destination of these herbs and liver, stomach, heart, lung and kidney ranked the second to the sixth. On the contrary, triple energizer, pericardium, gallbladder and bladder ranked at the end with the amount less than 10. Besides, we also observed the cross mapping of Cold/Hot properties and meridian (Fig. 4A), and it was worthy to be noticed that the most frequent pair was Warm and spleen. This finding was corresponding with previous hypothesis, since spleen is an important immune-related organ and one of the most important effect of Hot herbs is enhancing immune regulation. However, the distribution of Cold herbs seems to be more concentrated in stomach, kidney, liver, lung and heart rather than spleen.

Molecular features of targets of cold/hot herbs for CG

To address the complexity of herbal compositions, many herbs may target the same biomolecules, as shown by network target analysis. To distinguish the tendencies of Cold and Hot herbs, we implemented a 0.7 threshold (refer to Methods) and categorized the targets into three groups: specific to Hot herbs, specific to Cold herbs, and shared by both. Shared targets are those potentially targeted by multiple Hot and Cold herbs simultaneously (Figure S3A). Cold herb targets include biomolecules such as TNF, ILR1, VEGFA, TLR2, and IL2RG, suggesting their roles in anti-inflammation and immune regulation (Figure S3C). Conversely, targets of Hot herbs are involved in energy metabolism processes including steroids, hormones, 5-HT, SSTR, NOS, CRH, and GABA, and key immune response molecules like IL6R, CXCR1 (Figure S3D). Additionally, shared targets involve key biological process participants like IL1B, CD4, AR, ESR1, ESR2, NFKB1, TGFB1.

KEGG and GO enrichment were also performed on Hot herbs targets and Cold herbs targets. It was found that, in the enrichment result of Cold herbs targets, biological processes related to inflammatory response, cytokines, chemokines and immune cells represented by macrophages were significantly enriched, as well as inflammatory pathways including TNF, VEGF, HIF-1a signaling pathways. Apart from these immune-related pathways or biological processes, those related to lipid metabolism were also significantly enriched, like fatty acid oxidation and lipid oxidation (Fig. 4B). The enrichment result of Hot herbs targets was much different, which mainly fell in biological processes bound up with

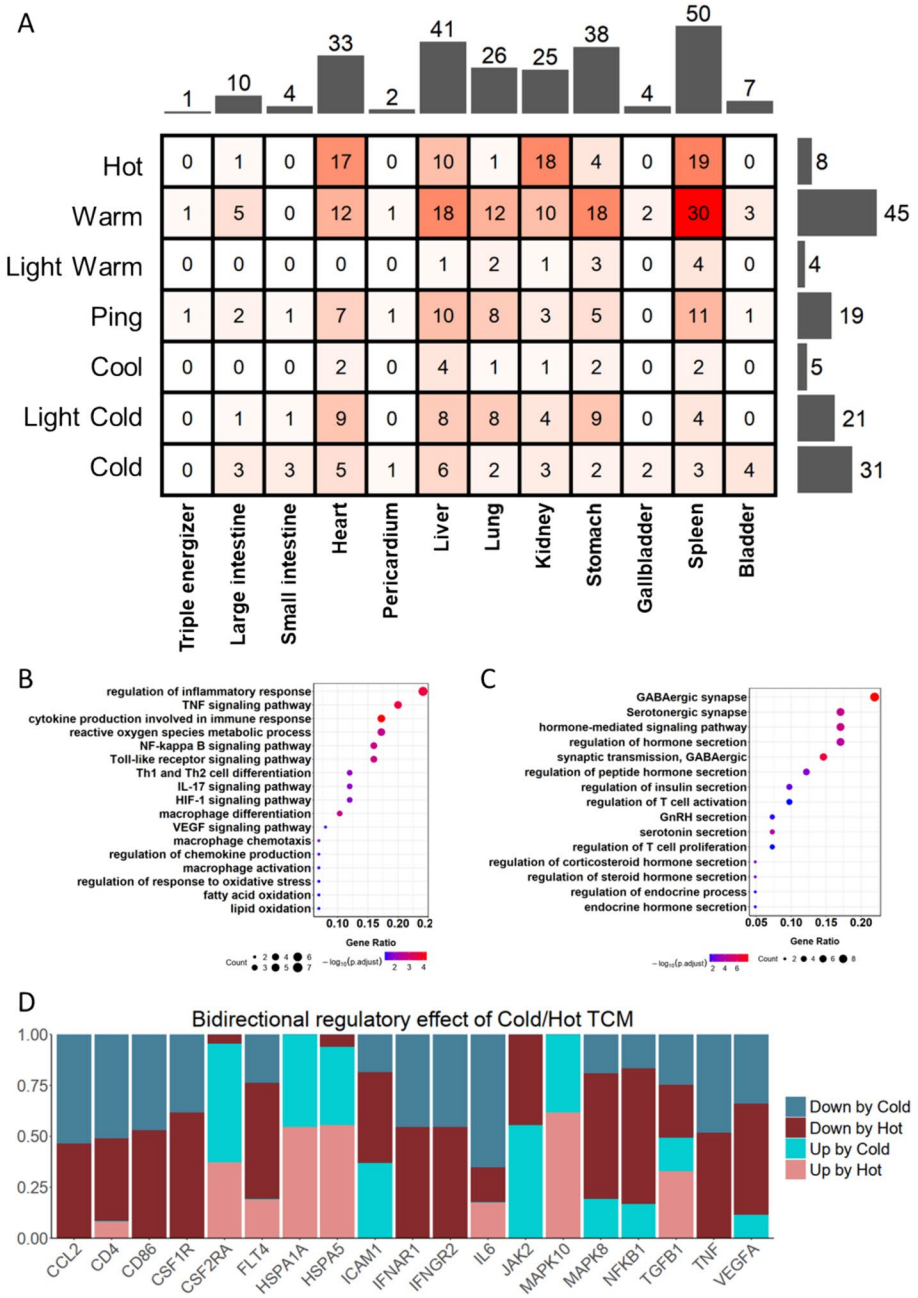


Fig. 4 Analysis of Cold/Hot herbs in formulae for CG. **A** Heat map showing the Cold/Hot properties and meridian of these Cold/Hot herbs. **B**, **C** Dot plot showing KEGG and GO enrichment of targets of Cold/Hot herbs. **D** Bar plot showing the proportions of the bidirectional regulatory effects of Cold/Hot TCM on representative targets

inhibitory neurotransmitter like 5-HT, GABA, hormone including steroid hormone, corticosteroid hormone, peptide hormone and other endocrine hormones (Fig. 4C). Besides, the result also included cellular processes of T cells, like T cell activation and proliferation. In general, these findings showed that in the one hand, the mechanism of Cold herbs against Hot ZHENG mainly fell in immune-related and inflammation-related factors, as well as metabolism like lipid metabolism to some extent. On the other hand, the treatment of Hot herbs against Cold ZHENG CG were mainly related to neurotransmitter and endocrine, which further proved the closer relationship between Cold ZHENG CG and

stress-induced factors, and showed therapeutic potential of Hot herbs in this regard.

Through public transcriptomics dataset of TCM intervened THP-1 cell lines in previous study [39], there were 21 Hot herbs and 17 cold herbs recorded in the dataset matched with the total 108 herbs for CG. Based on the transcriptomics data, it was possible for us to identify the regulatory direction of Cold/Hot herbs on these targets to some extent (Fig. 4D). It was found that targets like TNF, CCL2, CD86 and CSF1R showed consistently downregulation by both Cold/Hot herbs, and according to the literature, they played important roles in immune and inflammation regulation [40–43]. On the other hand, targets like HSPA1A and MAPK10 were both up-regulated by Cold/Hot herbs. Besides, targets like TGFB1, ICAM1 received up-regulation and down-regulation from many Cold/Hot herbs while IL6 and VEGFA were subject to bidirectional regulation but primarily down-regulated by both Cold/Hot herbs. In additional, SSTR2 were found to be decreased by Cold/Hot herbs (Figure S3E), which might lead to the change of Somatostatin (SS) [44, 45]. It was worth to be noticed that SS and its receptor played important roles in gastric diseases [46–48], and its absence might induce GC [49].

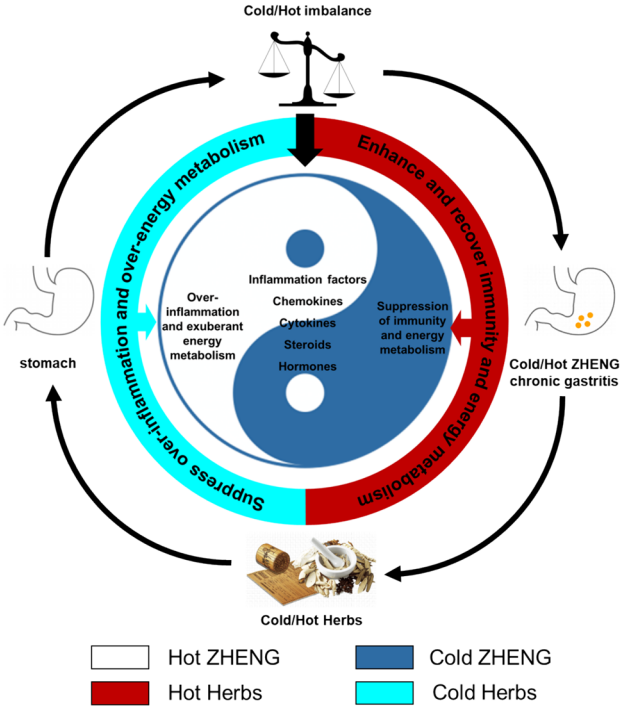


Fig. 5 Tai Chi Diagram showing the regulation programs of Cold/Hot herbs dominated by bi-directional regulation on inflammation/immune and energy metabolism. Cold herbs showed a trend to suppress the over-inflammation and over exuberant energy metabolism in Hot ZHENG, while Hot herbs preferred to enhance and recover immunity and energy metabolism in Cold ZHENG

Bidirectional regulatory effect of TCM in cold and hot CG

These results preliminarily revealed the mechanisms of action of Cold/Hot herbs in the treatment of CG associated with Cold/Hot ZHENG (Fig. 5). These functional characteristics of Cold/Hot herbs in CG formulae for immune regulation and metabolism regulation were also found to be therapeutic targets in researches of other formulae for other diseases [50–53].

Based on what we found for molecular features of Cold/Hot herbs, two specific networks for the Cold/Hot herbs targets respectively, according to the interaction recoded in STRING database (Figure S4). In Cold herbs targets, INS, TLR2, VEGFA, TNF, IL1R1 and TGFB3 showed a vital role in the targets network of Cold herbs for CG. And in Hot herbs targets, 5-HT related genes HTR1A and HTR1B, NOS1, GABA-related genes GAB family and CRH, as well as somatostatin receptor including SSTR2 and SSTR5 were found in the targets network of Hot herbs for CG. In other words, Hot herbs might regulate Cold ZHENG gastritis through regulating endocrine and energy metabolism. Apart from these genes, targets like CCL2, IL6, JAK2, IL2RA and CXCR1 were also of vital importance in the Hot herbs' targets network, which might infer us that another potential mechanism of Hot herbs against Cold ZEHNG gastritis was to regulate immune responses.

Immune-related pathways and biomolecules like TLR2 and CD14 were potential targets of Weifuchun capsule, which was clinically used for CAG, composed of Cold/Hot herbs and performed effect on both Cold/Hot ZHENG, like regulating immune response and anti-inflammation [54]. Huangqi Jianzhong decoction, a formula for Cold ZHENG CG, showed protective effects in CAG rats might be due to the balance of energy expenditure [55]. Taken together, the thermogenesis and immune-enhancing effects of Hot herbs may contribute to the therapeutic effect on Cold ZHENG CG. For herbs with cold property, their treatment of Hot ZHENG CG relies mainly on their anti-inflammatory effects, such as involving the TNF signaling pathway, NF-κB signaling pathway, and VEGF signaling pathway. Zuojin Pill is used in the treatment of Hot CG because it contains the cold property herb (Huanglian, dried rhizome of *Coptis chinensis* Franch., *Coptis teeta* Wall., and *Coptis deltoidea* C.Y.Cheng and P.K.Hsiao), which has been shown to have anti-inflammatory effects on CAG in rats by inhibiting the NF-κB signaling pathway [56]. Another widely used formula for CAG, Moluodan, was reported to reduce the inflammation level, as well as increasing lipid accumulation in MNNG-induced cells [57]. Serum levels of TNF-α, IL-8, and VEGF have been reported to be associated

with the severity of CG, the severe degree of neutrophil infiltration in CG, and the severity of precancerous lesions in the stomach, respectively [58]. Weiqi decoction formula has been reported to reduce VEGF levels in CAG rats [59]. The inhibition of the inflammatory response is the main therapeutic route for cold property herbs in the treatment of Hot ZHENG CG. Besides, leptin is considered to be a link between the neuroendocrine and immune systems and could be a possible target for intervention in immunometabolism-mediated pathophysiology and it has been reported that leptin resistance individuals have lower NK cell count and function than normal individuals [60]. Gastric mucosal leptin expression was significantly higher in *H. pylori*-positive patients than in negative patients [61]. Thus, in the context of immune-metabolic imbalances, leptin appears to be the pivotal molecule in the treatment of Cold/Hot CG with Cold/Hot herbs.

Transcriptomic level validation of the cold/hot regulatory effects of TCM

To demonstrate the role of TCM formulae in treating gastritis from the perspective of Cold and Hot, we selected a classic formula, Jin Hong tablets (a formula contains one Cold herb, Toosendan Fructus and three Warm herbs), which might have bi-directional regulatory effects from the perspective of Cold/Hot, constructed gastritis models and validated our findings through transcriptomics and molecular detection. Based on the differentially expressed genes identified in blood samples and gastric tissue samples (Fig. 6A, B), KEGG pathway enrichment analysis was performed on upregulated and downregulated genes (Fig. 6C). It was found that pathways related to Organismal systems, especially immune systems and endocrine system were significantly changed after the intervention of Jin Hong tablets. For example, estrogen signaling pathway were significantly enriched (Fig. 6D), and was reported to be of vital importance in both energy metabolism [62–64] and immune regulation [65, 66], which was consistent with our findings. More specifically, we observed the enrichment of differentially expressed genes in biological processes (Fig. 6E) and it was found that in gastric tissues, a significant number of metabolic-related biological processes underwent notable changes, including those related to peptides, glucose, and others. On the other hand, in blood, a significant number of immune-related biological processes were altered, including those related to immune response, cytokines, and various immune cells.

By deconvolving the transcriptomic expression matrix, we depicted the proportion changes of 6 major immune cells in gastric tissues before and after intervention, as well as in blood before and after intervention (Fig. 6F). For example, the proportions in CD8 + T cell and regulatory T cell undergone significantly changed after the intervention of Jin Hong tablets (Fig. 6G), which to some extent represented the restoration of immune response capabilities [67]. Finally, we measured the core targets in Cold/Hot biological networks, including IL-2, IL-6, SS and Leptin and found that their expression significantly recovered to normal levels (Fig. 6H).

Materials and methods

Differential analysis and PLS-DA for cold/hot ZHENG CG

According to the previous article, microarray data were collected from outpatients experiencing upper gastrointestinal discomfort undergoing endoscopic examinations at Beijing Dongzhimen TCM Hospital and Beijing Xiyuan TCM Hospital. Their histopathology was graded and classified as CSG or CAG based on the updated Sydney System [68, 69]. Two senior gastroenterologists specializing in TCM diagnosed the patients with either Cold ZHENG or Hot ZHENG.

To find the significantly differential expressed genes in Cold/Hot ZHENG CG, R package limma [70] were used to construct the generalized linear model. Genes with significant changes ($\log_2$(Fold Change) ≥ 1 or ≤ -1, adjust P value < 0.05, BH correction) were considered as differentially expressed genes in CAG and CSG, respectively. The DEGs in Cold/Hot ZHENG CG were defined as the DEGs in both Cold/Hot ZHENG CAG and CSG (adjust P value < 0.05, BH correction). PLS-DA (Partial Least Squares Discriminant Analysis) was performed based on R package mixOmics [71] v6.14.1. VIP (Variable Importance in the Projection) for seeds genes of Cold/Hot ZHENG was calculated to estimate the importance of each seeds gene in contributing to distinguishing Cold/Hot ZHENG CG (VIP > 1) with function PLSDA.VIP().

Enrichment analysis and immune characteristics of cold/hot ZHENG CG

To find the enriched pathways or biological processes which biomolecular features of Cold/Hot ZHENG CG involved in, enrichment analyses were performed based on R package clusterProfiler [72], including KEGG enrichment, GO enrichment and GSEA (Gene Set Enrichment Analysis). Pathways or biological processes significantly enriched (adjust P value < 0.05, BH correction) were kept for further analysis. Besides, for GSEA results, pathways or biological processes significantly enriched were further divided into 'active' or 'inhibit' based on their positive or negative NES (Normalized Enrichment Score) value.

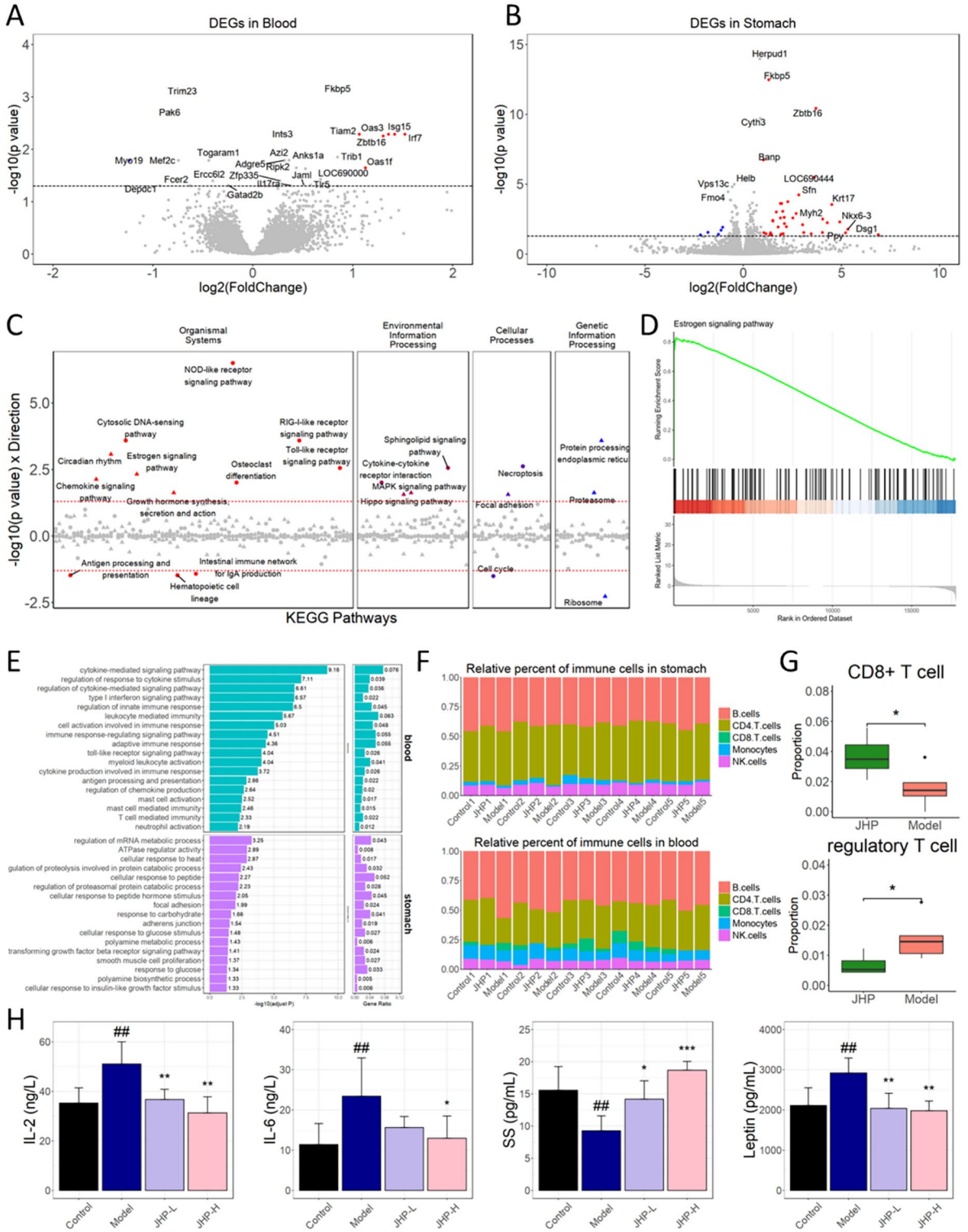


Fig. 6 Experiments validation from the perspective of Cold/Hot ZHENG. **A**–**B** Volcano plots for differential expressed genes before and after the intervention of Jin Hong tablets in blood and stomach, respectively. **C** Dot plot showing pathways with enrichment of genes differential expressed in blood and stomach, as well as their classification (dots below 0 were enriched by downregulated genes and dots above 0 were enriched by upregulated genes). **D** Representative enrichment of GSEA. **E** Enrichment result of the siginificantly changed pathways or biological processes in blood and stomach before and after the intervention of Jin Hong tablets. **F** Bar plot showing the proportions of immune cells in different samples before and after the intervention of Jin Hong tablets. **G** Inferred proportions of two types of T cells before and after the intervention of Jin Hong tablets. **H** Bar plot showing the expressions of IL-2, IL-6, SS and Leptin in blood

Targets prediction of formulae, herbs and corresponding compounds

In order to predict the potential targets of compounds in herbs of formulae for CG, based on our network-based computational algorithm, durgCIPHER-CS [14], the genome-wide targets and druggable targets were calculated. Top 100 targets of the druggable targets for each compound were considered as the target profile for each compound. A computational strategy [15] was performed to calculate the holistic targets of our collected formulae for CG and the Cold/Hot herbs that made up them. Targets with significant occurrence (adjust P value < 0.05, BH correction) were listed as the holistic targets of the herbs or formulae.

Definition of cold/hot herbs' targets and network construction

Taking the complex composition of herbs into consideration, many biomolecular might be the potential targets for many herbs. A strategy was implemented there to define targets for Hot herbs, Cold herbs and both Cold/Hot herbs. For each target, the counts of Cold/Hot herbs targeting on it were normalized on the total amount of Cold/Hot herbs and the ratio of the normalized counts of Cold/Hot herbs was used to divided targets into three groups, including targets of Hot herbs, targets of Cold herbs and shared targets. For Cold/Hot ZHENG CG, the biological network was constructed by the seeds genes with VIP value larger than 1 and DEGs in both CSG and CAG. Node side of each seeds gene depended on the VIP value. Besides, the biological networks for the targets of Cold/Hot herbs were constructed by the unions of targets of Cold/Hot herbs, respectively and seeds genes with VIP value larger than 1. All the gene–gene interactions in these networks were collected from STRING database [73].

Analysis of cold/hot TCM and traditional efficacy

To depict the potential mechanisms of formulae on features of Cold/Hot ZHENG CG, based on the holistic targets of formulae, KEGG and GO enrichment was performed through R package clusterProfiler (adjust P value < 0.05, BH correction). Six labels for traditional effects, including ZI YIN, XIAO JI, SAN HAN, QING RE, HUO XUE and XING QI could paste on these formulae according to TCM experience. And whether the label showed correlation with the composition of Cold/Hot herbs depended on Spearman correlation test (P value < 0.05).

Animal model construction and intervention

The animal experiment was conducted in accordance with the internationally accepted principles for laboratory animal use with the animal certificate number 2023100511 by Kangyuan Pharmaceutical Experimental Animal Ethics Committee. After adaptive breeding of the animals, except for the normal control group, the rest of the rats started continuous modeling for 42 days to establish an experimental CSG (chronic superficial gastritis) model in rats using combined factors: a mixture of 30% ethanol and 2% sodium salicylate solution, administered three times a week at a dose of 10 mL/kg by gavage; 10 mmol/L sodium deoxycholate, administered twice a week at a dose of 10 mL/kg by gavage; 0.05% ammonia water provided as the daily drinking water for free consumption by the rats; and irregular feeding patterns, with 2 days of full feeding followed by 1 day of fasting.

Then, medication was administered by gavage for 30 days (for the first 15 days in the morning, continuous modeling was carried out (for both the model group and various medication groups), and in the afternoon, the model group was given saline by gavage, while the medication groups were given low-dose Jin Hong tablets at 70 mg/kg and high-dose at 280 mg/kg (Jin Hong tablets, Batch number: 230602, provided by Jiangsu Kanion Pharmaceutical Co, Ltd). Food was withheld the day before tissue collection, but water was not.

RNA-seq sampling and analysis

Blood Samples: Blood is collected from the abdominal aorta, with 1 mL of blood transferred into an anticoagulant tube, then into a 15 mL centrifuge tube (pre-filled with 5 mL of Vezol Reagent). Stomach Tissue Samples: Take 20 mg of stomach tissue into a 1.5 mL centrifuge tube (pre-filled with 1 mL of AllProtect™ animal tissue nucleic acid and protein stabilizing solution).

RNA-seq analysis was conducted based on R package DESeq2 v1.30.0 and clusterProfiler v4.9.0. Significantly differential expressed genes (P value < 0.05) were kept for further analysis. Pathways and biological processes were enriched on KEGG, GO and GSEA. Immune cell fractions were inferred by CIBERSORT into 6 main classes and 22 sub classes with default parameters.

Serum biomolecular detection

On the day before the test, food was withheld but not water. The rats were anesthetized with 20% urethane (6 mL/kg), fixed in a supine position, and the abdominal skin was cut open to isolate the abdominal aorta for blood collection. The collected blood was then placed on ice, centrifuged 2 h later at 3500 rpm for 10 min. After obtaining the serum, it was aliquoted and stored in a − 80 °C freezer for subsequent detection using kits. Rat serum samples were tested for the contents of IL-6, IL-2, Leptin, and SS according to the instructions of the ELISA kits.

Discussion

Hot and Cold ZHENG is a dominating theory in TCM and they represent different conditions and phenotypes within one disease [74]. For example, in previous studies, significant differential symptoms in a cohort of cases with SARS were closely related to Cold ZHENG [75]. However, the mechanism of Cold/Hot ZHENG, as well as that of "Hot herbs for Cold ZHENG, Cold herbs for Hot ZHENG" remain unclear. In this study, on the basis of network target [76], we conducted a comprehensive study of Cold/Hot ZHENG CG and corresponding traditional formulae for gastritis composed of Cold/Hot herbs to uncover the mechanism of two traditional properties, Hot and Cold for diseases and herbs originating from ancient China.

Based on microarray datasets for CG of different ZHENG and advanced machine learning algorithms, we found the genes of vital importance as well as pathways or biological processes significantly enriched and constructed a biological network of seeds genes of Cold/Hot ZHENG and DEGs in CG of different ZHENG. It was found that pathways and biological processes related to immune and inflammation regulation were significantly active in Hot ZHENG patients and their related biomolecules were considered as hub nodes in this network due to their high degrees. Meanwhile, pathways and biological processes related to steroid and hormones were found to be active in Cold ZHENG and the corresponding biomolecules were also of vital importance in the network.

In general, the findings in this study could infer us that the main differences between Hot ZHENG and Cold ZHENG CG might be the over-inflammation in Hot ZHENG, as well as the suppression of immune and energy metabolism in Cold ZHENG. Consistent with our previous study that hormone-related biological processes are predominant in the Cold ZHENG network and immune-related biological processes are predominant in the Hot ZHENG network [11]. More specifically, they might suggest that there are different biological mechanisms between Hot and Cold ZHENG CG, implying that different specific treatment strategies are required for CG depending on ZHENG. Chemokines and cytokines like CCL2 were important biomolecules representing Hot ZHENG from the aspect of immune regulation and inflammation [8], while leptin and nitric oxide involved energy metabolism was another representative difference between Hot and Cold ZHENG.

Then, based on network analysis, we carried out network target analysis and described the holistic targets profiles of formulae for CG and their corresponding composition of Cold/Hot herbs. Apart from the target information, we also took meridian and Cold/Hot information into account to find the potential relationship between Cold/Hot properties and the tissues where herbs might take effect in. Targets of Cold/Hot herbs were divided into three groups and interestingly, it was found that targets of Hot herbs were significantly enriched in metabolism, regulation or endocrine of GABA, hormones, steroids and 5-HT, which were reported to be closely related to energy metabolism and thermogenesis, while targets of Cold herbs were mainly enriched in inflammation regulation like TNF, HIF-1, VEGF signaling pathway as well as immune response including cytokines, chemokines and cellular processes of immune cells. In our constructed biological targets network for Cold/Hot herbs against CG, biomolecules related to inflammation and immune regulation like TNF, TLR2, TGFB3, IL2RG, IL1R1 and VEGF were of vital importance for Cold herbs, while those involved in energy metabolism like SSTR, HTR, GABA and CRH, as well as those related to immune response like CCL2, IL6, IL2RA, IL2RB and JAK2 played important roles in the network for Hot herbs.

Considering the difference in the immune status of Hot and Cold ZHENG CG and the risk of transformation of CG into cancer, we analyzed and compared the association between Cold/Hot ZHENG CG and Cold/Hot tumors. The hot tumors are characterized by immune activation with T cell infiltration, whereas Cold tumors show lack of T cell infiltration or absence [77]. In this study, we found that the biological processes related to immunity, inflammation, cytokines and chemokines were activated in patients with Hot ZHENG CAG and inhibited in patients with Cold ZHENG CAG. Some key seeds genes of Hot/Cold ZHENG play an important role in converting cold tumors into hot tumors through increased T-cell infiltration, such as TGFB, CCL2, VEGF, and TLR [78]. TGFB is an immunosuppressive molecule, and its inhibition increases T cell infiltration [79]. Loss or low expression of specific chemokines and their corresponding receptors, such as CCL2 and CCL5, reduces infiltration of effector T lymphocytes [80]. It has been reported that VEGF could interrupt T-cell priming, inhibit DC maturation and exhaust CTLs [81]. Thus, the recognition of Cold/Hot ZHENG of tumors, such as gastric carcinoma, is particularly important due to the different immune modulation by Cold/Hot herbs treatment.

Last but not least, in order to achieve personalized and precise medical treatment, precision medicine [82], as well as precision TCM [83] which originates from it, provides a new insight for present medical strategy. The diagnosis of Cold/Hot ZHENG is a holistic observation which potentially represents the states of immune regulation and energy metabolism for patients. And decision on Cold/Hot herbs based on corresponding Cold/Hot ZHENG may be a kind of precision TCM based on

the macroscopic phenotypes combined with traditional experience. With the help of research to uncover the mechanism of Cold/Hot ZHENG and herbs, the potential law of precision TCM from the perspective of Cold and Hot may be revealed and thus facilitate newer and more precise medical strategy.

There were still some limitations in this study. Firstly, we summarized the mechanism of Cold/Hot ZHENG CG and Cold/Hot herbs for CG formulae to the level of pathways and biological processes, combined with present studies and proposed some potential biomarkers of these mechanisms. However, these biomarkers haven't been verified in detail. Then, compounds of each herb in the formulae for CG were collected from databases for TCM. However, the recorded data in these databases might not be as accurate as the result detected by high-performance liquid chromatography analysis or other detection analysis. Besides, in the perspective of formulae, we haven't completely uncovered the mechanism for each of them due to their complex composition and some of them were composed of both Hot and Cold herbs, forming complicated effect on chronic gastritis. Fortunately, these shortcomings of data constitution could be partly made up by our network-based algorithms and network target analysis. And the further analysis of each formulae might need be treated and analyzed respectively to reveal the comprehensive and specific mechanism for them each.

In conclusion, from two starting points, we conducted exhaustive analysis to find vital biomolecules and biological features for Cold/Hot ZHENG CG and Cold/Hot herbs for CG based on the combination of gene expression data, network analysis, statistic models and machine learning algorithms. And it was found that two specific characteristics between Hot and Cold ZHENG were the differences in immune and inflammation responses, as well as those in endocrine, energy metabolism, and thermogenesis. In general, Hot ZHENG CG showed a trend of over-inflammation and exuberant energy metabolism, and that in Cold ZHENG CG was the suppress of immune regulation and energy metabolism. Besides, in the aspect of Cold/Hot herbs, Hot herbs preferred to target on the biomolecules or biological processes of immune response and energy metabolism, while Cold herbs had the potential effect on inflammation and immune regulation. This study didn't only uncover the potential mechanism of Cold/Hot ZHENG CG and Cold/Hot herbs in formulae for CG, but also might potentially provide a new insight for diagnosis of Cold/Hot ZHENG in diseases and further offer better and more precise medication strategies for Cold/Hot ZHENG patients to achieve precision TCM in the treatment of diseases like gastritis.

Abbreviations

CAG	Chronic atrophic gastritis
CG	Chronic gastritis
CSG	Chronic superficial gastritis
DEGs	Differentially expressed genes
PLS-DA	Partial least squares discriminant analysis
TCM	Traditional Chinese medicine
VIP	Variable importance
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene ontology
GSEA	Gene set enrichment analysis
scRNA	Single-cell RNA
SS	Somatostatin

Supplementary Information

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Supplementary Material 1.

Author contributions

Conceptualization, S.L.; supervision, S.L. and T.Z.; experimental design, T.Z.; investigation, B.W. and P.C.; data curation, B.W. and P.C.; formal analysis, B.W. and T.Z.; experimental execution, L.X.; writing—original draft preparation, B.W. and P.C.; writing—review and editing, S.L., H.C., L.C. and P.Z.; visualization, B.W. and Z.Z. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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