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Integrated analyses of transcriptomics and network pharmacology reveal leukocyte characteristics and functional changes in subthreshold depression, elucidating the curative mechanism of Danzhi Xiaoyao powder

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Abstract

Purpose: to learn more about the molecular basis of subthreshold depression (SD), find medications that might help with it, and understand how Danzhi Xiaoyao powder (DZXY) works in SD. Methods: We used RNA-sequencing to find the hub genes of SD, roles and pathways of differentially expressed genes (DEGs) in SD leukocytes compared to healthy controls, and to identify these genes. Using the TELiS technology, we also evaluated alterations in leukocyte transcription factor activity in SD patients. Potential SD medications were screened by retrieving the Connectivity Map information. We used network pharmacology to explain how DZXY works to treat SD by identifying its "multi-component, multi-target, and multi-pathway" mechanism.

Found 1080 differentially expressed genes (DEGs) in the white blood cells of SD patients ($p < 0.05$, $|\log2(\text{fold change})| \geq 1$, and protein coding). Immune and inflammatory response-related activities were the primary domains of activity for these DEGs, which included hub genes. A comparison of the SD leukocyte transcriptome profile with the conserved immune cell transcriptional response to adversities was shown by transcription factor activity analysis. Among the 28 medications that might be useful in treating SD, the Connectivity Map analysis highlighted SB-202190 and TWS-119. The therapeutic mechanisms of DZXY in SD, mainly encompassing in-flammatory response, lipid metabolism, immunological response, and other processes, were discovered by constructing the "Direct Compounds-Direct Targets-Pathways" network for both DZXY and SD.

1. Introduction

A number of mental and physical health issues might worsen symptoms of major depressive disorder (MDD) and other mental diseases. The Report on National Mental Health Development in China (2021e2022) states that psychological risk has surpassed physical danger to become one of the top ten global hazards, with depression being a leading cause of disability worldwide.1 In subthreshold depression (SD), people show signs of depression but do not yet meet the diagnostic criteria for a full-blown depression. This is because the severity and length of depressive

symptoms do not meet the current standards.2 People with SD nevertheless have impaired physical and mental health, much like those with MDD, even if their symptoms are less severe and don't last as long. When compared to healthy persons, SD groups also have a greater chance of

getting MDD or other mental illnesses.3 Research and clinical diagnosis have paid little attention to SD because of the dominating categorical diagnostic paradigm for mental diseases. The recently published presentation of a depression spectrum due to SD's significant functional impairment, which calls for more research into the disease's pathophysiology, diagnostic tools, and treatment strategies. It is critical to study the pathophysiological relationship between SD and MDD.4e7 Two of the most well acknowledged molecular processes of depression in the last 20 years have been elevated inflammation and hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis.8 A confirmed pattern change in the gene expression profile of immune cells has been seen in both people and animals as a result of long-term exposure to unfavourable environmental circumstances, including various socioenvironmental risk factors (a significant class of variables that might cause depressive disorders). Genes associated with inflammation are increased while genes connected to the innate antiviral response are downregulated; this altered pattern is called the conserved transcriptional response to adversity (CTRA).9 The intricacy of the CTRA, together with individual differences in genetics, psychology, socioenvironmental factors, and other factors, makes it impossible to develop a foolproof technique for measuring the CTRA. Nevertheless, by employing a range of bioinformatics techniques, including DEG biofunction tagging, analysis of transcriptional control pathways, quantification of myeloid cell population dynamics, evaluation of immune function, and evaluation of a priori-defined gene sets, several important components of CTRA were effectively utilised, yielding reliable indicators for CTRA evaluation. As far as CTRA measurement is concerned, the most sensitive and trustworthy method is transcription factor activity analysis based on genome-wide transcriptome differences.9 Enhanced activation of the SNS (represented by the CREB/ATF transcription factor family), enhanced activation of proinflammatory factors (e.g., the NF-kB/Rel family), decreased activity of interferon response factors (IRFs), and impaired glucocorticoid receptor (GR) activity were all revealed by these multilevel bioinformatic analyses. Extensive research has shown that the CTRA pattern or its properties are present in the immune cell gene expression profiles of humans with depression, those suffering social stress, and analogous animal models using these different methodologies.9e12 Given that SD is often accompanied with depression, we postulated that circulating immune cells from SD patients would have a gene expression profile similar to the CTRA pattern.

The provision of medicinal interventions for SD is fraught with ambiguity as a result of differing perspectives. The safety and effectiveness of current antidepressants and other antidepressant treatments have not been well evaluated, and there have been few trials on SD-specific treatments. According to the theory of syndrome and disease differentiation in traditional Chinese medicine (TCM), SD and depression are emotional illnesses that fall under terms like "depression," "visceral mania," "lily disease," and "sleeplessness." One common symptom of these conditions is liver-qi stagnation. Therapeutics for SD should aim to target many pathways at once due to the complexity of the disease's pathophysiology. Chinese herbal formulae naturally provide the benefits of a wide variety of active substances, as well as actions that target many channels at once. Among the ten traditional Chinese medicines that make up Danzhi Xiaoyao powder (DZXY), you'll find Bupleurum chinense DC., Angelica sinensis (Oliv.) Diels, Paeonia lactiflora Pall., Atractylodes macrocephala, and Dang Gui. Koidz. (A. macrocephala, Bai Zhu), Glycyrrhiza uralensis Fisch. (G. uralensis, Gan Cao), Paeonia suffruticosa Andr. (P. suffruticosa, Mu Dan Pi), Gardenia jasminoides Ellis (G. jasminoides, Zhi Zi), Mentha haplocalyx Briq. (M. haplocalyx, Bo He), and Zingiber officinale Rosc. (Z. officinale, Sheng Jiang). It is a typical traditional Chinese medicine prescription for the treatment of depression.13 In clinical and experimental settings, it has been used either alone or in combination with antidepressants by clinicians and researchers.

research of depressive disorders, demonstrating positive thera- peutic effects, fewer side effects, and better patient compliance. However, limited research has evaluated the efficacy and mecha- nisms of DZXY in the treatment of SD, hindering its promotion and application.

Thus, based on the popular RNA-sequencing (RNA-seq) approach, in this study we first attempted to identify the mRNA expression signature in the circulating immune cells of

SD and subsequently investigated whether these specific SD transcription profiles shared the CTRA characteristics. SD transcriptomic data were also used, in conjunction with the Connectivity Map database (CMap, https://clue.io, accessed May 28, 2023), to identify candi- date small molecule drugs for SD.[14] With CMap providing targets and the mechanisms of action (MOA) of small-molecule com- pounds, we attempted to further decipher the pathogenesis of SD. To provide theoretical and experimental support for the clinical application of DZXY in the treatment of SD and further elucidate its anti-depressive mechanisms, we investigated its "multi-compo- nent, multi-target, and multi-pathway" curative mechanism using a network pharmacology analysis strategy.[15,16]

2. Materials and methods

2.1. Ethical approval

The trial was registered with ChiCTR (ChiCTR2000032005). The study protocols were approved by the Ethics Committee of the Beijing University of Chinese Medicine (2020BZYLL0605). All pro- cedures were performed in accordance with the relevant guidelines and regulations. The participants were informed of the purpose, general content, and data use of the study, and provided written informed consent.

2.2. Participants

Patients with SD were recruited from the Third Affiliated Hos- pital of Beijing University of Chinese Medicine, and healthy controls from the Beijing region were matched for sex, ethnicity, and age (Supplemental Table 1). All the participants were recruited using bulletin board notices, WJX posts (https://www.wjx.cn, accessed October 1, 2021), and clinical referrals. The diagnosis of SD followed the currently accepted diagnostic criteria. First, the Mini- International Neuropsychiatric Interview (MINI) was used to determine whether participants exhibited depressive symptoms leading to functional impairment, yet did not meet the diagnostic criteria for major depressive episodes in the *Diagnostic and Statis-*
tical Manual of Mental Disorders (Fifth Edition). Second, participants had to score ≥16 on the Center for Epidemiological Studies Depression Scale. Concurrently, trained psychiatrists conducted face-to-face interviews and used the 17-item Hamilton Depression Rating Scale (HAMD-17) to reconfirm the eligibility of participants' (7 ≤ HAMD-17 < 17).[2,17,18] The experimental design (Fig. 1), including complete inclusion and exclusion criteria for all the par-
ticipants in this study, is detailed in the supplemental information (Supplemental Data).

2.3. Leukocyte isolation and RNA extraction

Following an overnight fast, blood samples (3 mL) were collected from individuals via venipuncture into vacutainer tubes containing EDTA between 7:30 AM and 8:30 AM. Blood samples
(3 mL) were subsequently transferred into centrifuge tubes and centrifuged at 550×*g* for 30 min at 25°C, and the supernatant (plasma) was collected and preserved at —80°C for subsequent studies. The remaining cell precipitates were used to isolate

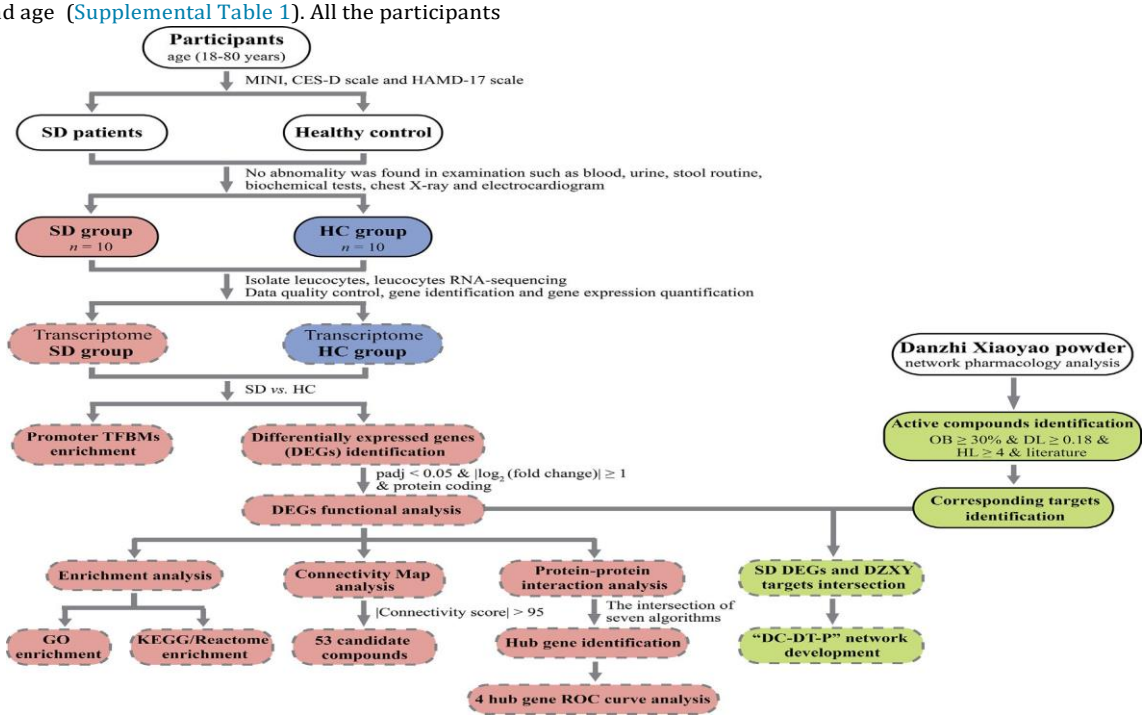


Fig. 1. Experimental design and study route.
Notes: MINI: Mini-International Neuropsychiatric Interview; ROC: receiver operating characteristic; OB: oral bioavailability; DL: drug-likeness; HL: half-life; DT: direct target of Danzhi Xiaoyao powder in subthreshold depression; DC: DT corresponding active compounds in Danzhi Xiaoyao powder; P: KEGG pathways and GOBP annotation items; HC: healthy control; SD: subthreshold depression.

leukocytes using red blood cell lysis buffer (Solarbio, Beijing, China) according to the manufacturer's instructions. Finally, the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) was used to
extract the total RNA from leukocytes following the manufacturer's instructions and stored at —80°C.

2.4. RNA sequencing

RNA sequencing of leukocyte samples was performed using the Novogene Company (Beijing, China). The total amount and integrity of the RNA were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). mRNA was purified from the total RNA using poly T oligo-attached magnetic beads (New England Biolabs, Ipswich, MA). All tran- scriptomic sequencing libraries were constructed using the NEB- Next Ultra RNA Library Prep Kit (NEB #E7770; Illumina, San Diego, CA). The resultant libraries were sequenced using the Illumina sequencing platform (Illumina NovaSeq 6000), and 150 bp paired- end raw reads

Differentially expressed gene identification

Gene expression levels were standardized using FPKM (frag- ments per kilobase of the exon model per million mapped reads). Differential expression analysis of the SD/HT groups was performed using the DESeq2 R package 1.20.0.[19] The resulting *P*-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate (represented in the form of padj). "Padj
<0.05 & $|\log_2$ (fold change)$| \geq 1$ & protein coding" were set as the
thresholds for DEGs.

2.5. Expression pattern clustering

Hierarchical clustering (HCL)-based expression analyses of DEGs were performed using the pheatmap R package 1.0.12, and the reshape2 package 1.4.4 was combined to generate HCL-analysis- based heat maps.[20]

2.6. Function and pathway enrichment analysis

2.7. databases such as Reactome (https://reactome.org, accessed April 20, 2022), the Kyoto Encyclopaedia of Genes and Genomes (KEGG, https://www.kegg.jp, accessed April 20, 2022), and others... To directly detect the DEGs' highly enriched GO and route keywords, the clusterProfiler R package 4.3.4 was used.21

3.7. Analysing transcription factors

To determine TF activity, the TELiS promoter-based bioinformatics analysis platform was used to identify all genes in both groups (http://www.telis.ucla.edu, accessed August 28, 2022).22 The idea of "gene prompter TF-binding motif (TFBM) enrichment" is central to this TF activity analysis; in this model, active TFs alter gene expression in response to TF binding sites. Promocer TFBMs for active TFs were therefore more abundant in the upregulated gene subset compared to the control gene set. The supplementary materials (Supplemental Data) describe in depth the procedures and statistical approaches used in the analysis. In this study, we utilised the gene promoter TFBM enrichment analysis results that met the criteria of "N (ratios analysed times) ¼ 9 & Boot- strap P <.05" to assess the functional conditions of five transcription control pathways. These pathways include the CREB/ATF family, the NF-kB/Rel family, the IRF family, GR, and Nrf2 mediating signalling pathways. These pathways have been linked to the development of MDD and have revealed the basic mechanisms of CTRA (with the exception of the Nrf2 contro

The 3.8. Hub gene receiver operating characteristic curve analysis, subthreshold depression-related hub gene discovery, and interaction network analysis

A cut-off threshold with a combined score of $\geq$0.7 was used to analyse both direct and indirect associations among the DEGs, and the interaction network of the relevant proteins was built using the online STRING database 12.0 (https://cn. string-db.org, accessed May 30, 2022).25 Afterwards, the gained complex protein-protein interaction (PPI) network was visualised and adjusted using Cytoscape 3.8.1 (https:// cytoscape.org, visited April 1, 2023) software. The cytoHubba 0.1 plug-in for Cytoscape was used to identify hub genes associated to SD. Within the cytoHubba plugin, seven common algorithms were used to generate seven sets of the top twenty hub genes: MCC, MNC, Degree, Closeness, Radiality, Stress, and EPC. The hub genes linked to SD were produced by the meeting of these seven categories.26, 27 Finally, to assess the diagnostic significance of the discovered hub genes as biomarkers for SD, we constructed the receiver operating characteristic (ROC) curve using Prism 9.2.0 (GraphPad Software, San Diego, CA).

3.9. Analysing the Connection Map

Using at least three distinct types of grown cells, the Connectivity Map looked at how over 1,400 bioactive chemicals affected

mRNA expression levels throughout the world. In order to compare the compound-specific mRNA expression signatures with the mRNA expression signatures of interest, we used the "Query" feature of the most recent CMap database (https://clue.io/query, accessed May 28, 2023) and calculated the correlations between them using tau (t) scores.13 The tau score is an improved iteration of the Connectivity Score that has been calibrated against the whole reference library backdrop, and then normalised across different drug kinds and cell lines.13,28 In this case, we used the "Query" to load the 400 most important SD DEGs, including 200 upregulated genes and 200 downregulated genes.

tool. We screened for Connectivity Scores (tau scores) < —95 to find small-molecule therapeutic candidates for SD, and we examined the molecular orbital alignment (MOA) of compounds with Connectivity Scores (tau scores) > 95 to illustrate the pathophysiology of SD.28
In order to find medications with similar or different expression profiles, the CMap "Query" tool could only examine up to 150 down- or up-regulated "Best Inferred Genes (BING)" at a time due to technological restrictions. All genes not included in the "BING" subgroup were not considered.13 The top 400 SD-expressing DEGs and compound-mediated alterations in neural progenitor cells (NPC) and developed neurons (NEU) were not analysed to screen prospective medicines, despite the tight biological link between SD and the nervous system. Cancer cell lines tend to be very homogeneous, however NPC and NEU cells are incredibly diverse in their molecular and transcriptome makeup. This suggests that some chemicals induce different gene expression in neuronal cell lines compared to homogeneous cancer cell lines. This might be due to biological differences between the two types of cells or to the compounds themselves.13%, 29%

Network pharmacology analysis

The information on the small molecular components in DZXY was retrieved from the following sources: the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP), the Traditional Chinese Medicines Integrated Database (TCMID), and literature retrieval.30,31 Our analysis of DZXY's active ingredients was guided by pharmacokinetic criteria such as drug-likeness (DL) > 0.18, half-life (HL) ≥ 4, and oral bioavailability (OB) ≥ 30%. Based on proven antidepressant action revealed in prior studies, DZXY also includes active components of many drugs that did not meet these standards. Based on human protein information in the UniProt Knowledge Base (https://www.uniprot.org/, accessed March 19, 2023), we were able to identify probable targets for the active components of DZXY using the TCMSP and TCMID. We next got their coding gene symbols. We created a "Compounds-Targets (C-T)" network diagram of DZXY by entering its active chemicals and possible targets into Cytoscape. Then, to find the specific genes that DZXY should target in order to cure SD, we crossed the SD DEGs with the DZXY active component targets. Direct compounds (DC) are the active ingredients that match these intersecting targets. We annotated GO biological processes, analysed KEGG pathways, and performed correlation analyses for the DTs using the ClueGO plug-in of Cytoscape.32 Lastly, a "Direct Compounds-Direct Targets-Pathways (DC-DT-P)" three-layer network was built and visualised using ClueGO and CluePedia, another Cytoscape programme, to show the underlying therapeutic processes of DZXY in SD.33

Results

2.8. Subthreshold depression differentially expressed genes and expression pattern clustering

Compared with healthy controls (n ¼ 10), 1080 DEGs were identified in the leukocytes of patients with SD (n ¼ 10), 744 of which were upregulated and 336 downregulated (Supplemental Fig. 1A, Supplemental Table 3). We subsequently performed HCL analysis of the expression profiles of leukocyte DEGs from these two groups. The resulting heat map displayed distinct clustering of expression profiles among individuals within the same group,

clearly distinguishing them from those in the other groups (Supplemental Fig. 1B).

2.9. GO functions of subthreshold depression differentially expressed genes

GO function-based enrichment analyses of these SD-related differential genes demonstrated that the immune system may be activated in patients with SD. The GO biological process (GOBP) analysis, with a gene count of ≥3 and $P < .05$, indicated that SD- related leukocyte DEGs were primarily associated with response
to stimuli. This included responses to lipopolysaccharide (GO: 0032496), response to molecule of bacterial origin (GO: 0002237), and cellular response to biotic stimulus (GO: 0071216). The enriched GO molecular function (GOMF) terms were primarily involved in cellular signal transduction, including cytokine activity (GO: 0005125), signaling receptor activator activity (GO: 0030546), DNA-binding transcription

activator activity (GO: 0001216), and G- protein-coupled chemoattractant receptor activity (GO: 0001637). Moreover, the GO cellular component (GOCC) terms indicated that these enriched genes encoded endocytosis- and efflux-related cellular structures, including tertiary granules (GO: 0070820), specific granules (GO: 0042581), and phagocytic vesicles (GO: 0045335). The top 10 significant GOBP, GOMF, and GOCC terms annotated by SD differentially expressed genes are shown in Fig. 2A and Supplemental Table 4.

2.10. Enriched pathways of subthreshold depression differentially expressed genes

KEGG and Reactome pathway-based enrichment analyses were performed to identify the potential pathways of SD-related differ- ential genes. The results revealed 31 SD-related DEGs enriched in KEGG pathways (gene count ≥3 and $P < .05$), including signal transduction (NF-kappa B signaling pathway, tumor necrosis factor
(TNF) signaling pathway, MAPK signaling pathway, and leukocyte trans-endothelial migration), infectious diseases (legionellosis, malaria, pertussis, leishmaniasis, etc.), immune system (IL-17 signaling pathway, chemokine signaling pathway, Toll-like receptor signaling pathway, etc.), and immune disease (rheumatoid arthritis) among others (Fig. 2B and Supplemental Table 5).
The 74 enriched Reactome pathways (gene count ≥3 and $P < .05$) showed that the SD-related differential genes in leukocytes
were enriched in pathways related to the immune system and immune system diseases (interleukin-4 and interleukin-13 signaling, neutrophil degranulation, interleukin-10 signaling, dis- eases associated with the TLR signaling cascade, reactive oxygen species (ROS) and reactive nitrogen species (RNS) production in phagocytes, etc.), nerve growth factor (NGF) signal transduction including signaling by NTRKs, signaling by NTRK1 (TRKA), nuclear events (kinase and transcription factor activation), NGF-stimulated transcription, and other pathways. The top 20 significantly enriched Reactome pathways are displayed in Fig. 2C, and complete enriched pathways are shown in Supplemental Table 6.

2.11. Transcription factor activity in subthreshold depression patients vs. healthy controls

Promoter TFBM enrichment analysis results (Fig. 3 and Supplemental Table 7) showed the activity of the selected TFs implicated in the pathogenesis of MDD, providing insights into the basic mechanisms of CTRA in patients with SD *vs.* healthy controls. Specifically, there was significant overrepresentation of response elements for CREB/ATF factors (VATF_01, VCREBP1_Q2, V$CREB_Q4, V$CREB_01, V$CREB_02, V$CREB_Q2, V$CREBP1CJUN_01), NF-kB/Rel factors (V$CREL_01, V$NFKB_C, V$NFKB_Q6, V$NFKAPPAB_01, and V$NFKAPPAB65_01) and the master antioxidant TF Nrf2 (V$NRF2_01) in the promoters of genes upregulated in association with SD. TFBMs targeted by GR (V$GR_Q6) and interferon-responsive TFs (V$ISRE_01) were significantly overrepresented in the promoters of downregulated genes associated with SD. This suggests that the pathological mechanism of SD may be associated with higher activity of CREB/ATF, NF-kB/Rel, and Nrf2 and lower activity of GR and IRF.

2.12. SD differentially expressed gene PPI network construction, SD hub gene identification, and hub gene receiver operating characteristic curve analysis

To identify the hub genes, a PPI network was constructed and visualized using the STRING online database and Cytoscape soft- ware. A total of 463 nodes and 789 edges were identified in the PPI network (Fig. 4A). Next, using the seven algorithms of the Cyto- scape plug-in cytoHubba, we identified the top 20 hub genes (Supplemental Table 8). Upon intersecting the results from the 7 groups of hub genes, 4 common hub genes were identified (Fig. 4B and Supplemental Table 8): TNF, interleukin 1 beta (*IL1B*), C-X-C motif chemokine ligand 8 (*CXCL8*), and early growth response 1 (*EGR1*).

Compared to healthy control participants, the expression of the hub genes *TNF*, *IL1B*, *CXCL8*, and *EGR1* was upregulated in the leukocytes of patients with SD (Supplemental Fig. 2). The ROC curve analysis results showed that the areas under the ROC curve (AUCs) of *TNF*, *IL1B*, *CXCL8*, and *EGR1* were 1.00 (95% CI: 1.00 to 1.00, $P < .001$), 0.81 (95% CI: 0.5942 to 1.00, $P < .05$), 1.00 (95% CI:
1.00 to 1.00, $P < .001$), and 0.87 (95% CI: 0.7092 to 1.00, $P < .01$),
respectively (Fig. 4C). The corresponding cutoff values were 152.2 (sensitivity, 100%; specificity, 100%), 1649 (sensitivity, 70%; spec-
ificity, 100%), 15 975 (sensitivity, 100%; specificity, 100%), and 4736 (sensitivity, 90%; specificity, 80%). Generally, an AUC ranging from
0.7 to 0.9 for a biomarker indicates some diagnostic value, and an AUC > 0.9 is considered highly valuable for diagnosis.[34,35] Notably, the sample size of this study was small, and the AUC of *TNF* and *CXCL8* was 1.00, indicating perfect classifiers, an outcome nearly impossible for continuous variables. Therefore, further verification with a larger sample size is necessary in future studies.

2.13. Connectivity Map analysis for subthreshold depression candidate drugs

Using Connectivity Map query results for the top 400 significant DEGs (200 increased and 200 decreased in SD), we identified 53 compounds. Among these, 28 exhibited summary Connectivity Scores in nine cell lines below —95, suggesting potential benefits in treating SD. The remaining 25 compounds with Connectivity Scores
over 95 in the nine cell lines and their targets and mechanisms of action may indicate the pathogenesis of SD. The exact Connectivity Scores for each cell line and their summary scores are shown in Fig. 5A and Supplemental Table 9.

The 28 candidate compounds for SD treatment included 20 mechanisms of action, such as phenylalanyl tRNA synthetase in- hibitors, glycogen synthase kinase inhibitors, JNK inhibitors, and CDK inhibitors (Fig. 5B and Supplemental Table 10). Nine of the 28 compounds, including fludarabine, daunorubicin, and dactinomy- cin, have been approved for clinical use in hematologic malig- nancies and oncology treatment. Veliparib, triptolide, saracatinib, and 7 other compounds are currently undergoing clinical trials, whereas PJ-34, TWS-119, and 6 others are in the preclinical study stage (Fig. 5A and Supplemental Table 10). Notably, SGK1 and JUN,

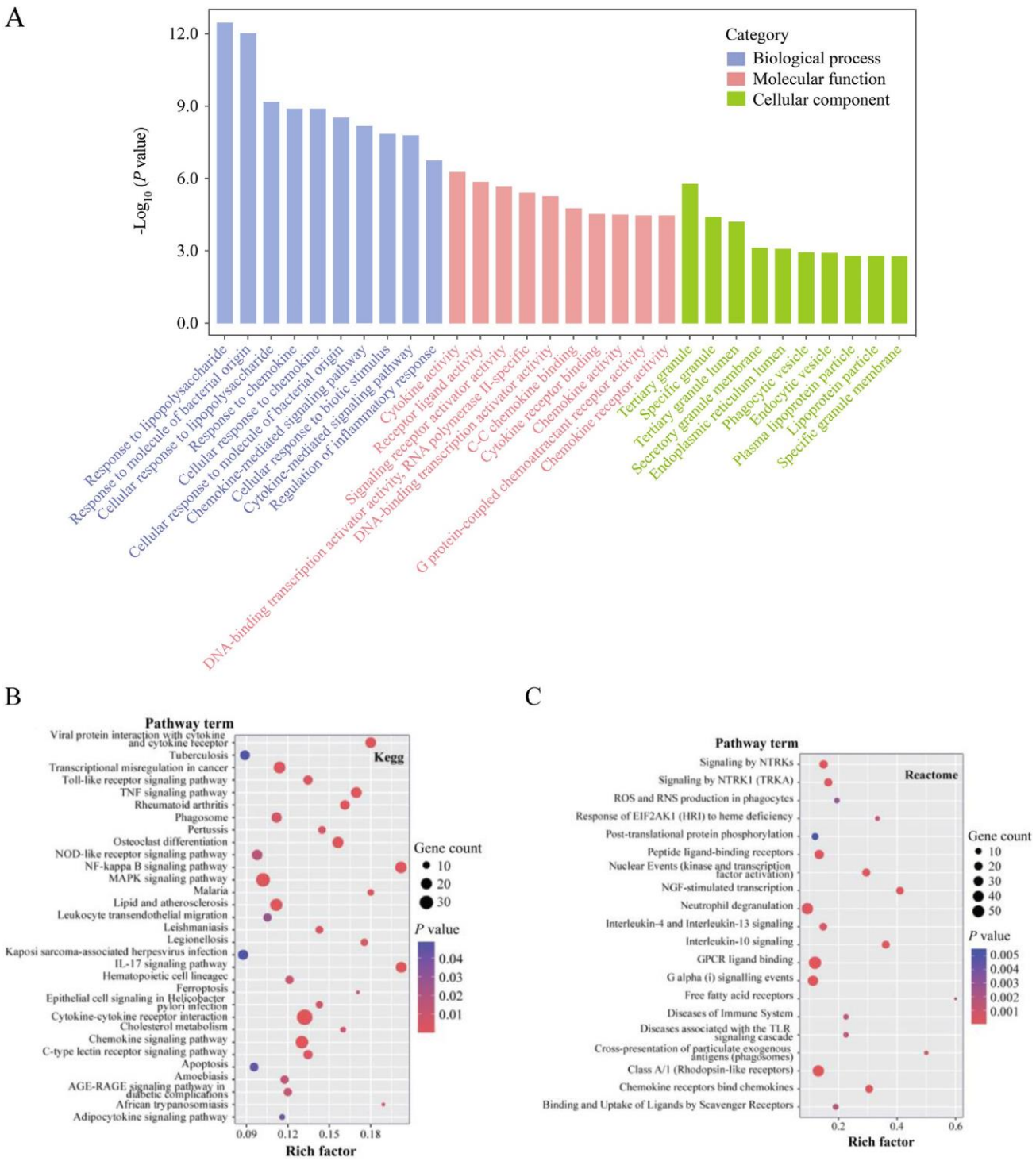


Fig. 2. Biological function analysis of DEGs in patients with SD: top 10 GO terms (BP\MF\CC) annotated by DEGs (A); bubble plot for KEGG enrichment pathways of DEGs (B); bubble plot for the top 20 Reactome enrichment pathways of DEGs (C).
Notes: In A, blue bars indicate the top 10 GO biological process terms, red bars indicate the top 10 GO molecular function te rms, and green bars indicate the top 10 GO cellular component terms. In C, the rich factor represents the ratio of DEGs annotated to a pathway to all genes annotated to that pathway. A smaller *P* value indicates greater pathway enrichment.

two of the 1080 SD differentially expressed genes, encoded direct targets for SB-202190 (MOA: p38 MAPK inhibitor) and TWS-119 (MOA: glycogen synthase kinase inhibitor), respectively (Supplemental Table 10). An additional 25 small molecular compounds with Connectivity Scores >95 included a PKC activator, M5 modulator, CCK receptor antagonist, and 10 other mechanisms of action (Fig. 5B and Supplemental Table 10). As shown in Fig. 5A and Supplemental Table 10, 10 launched drugs, including ouabain, mebendazole, and

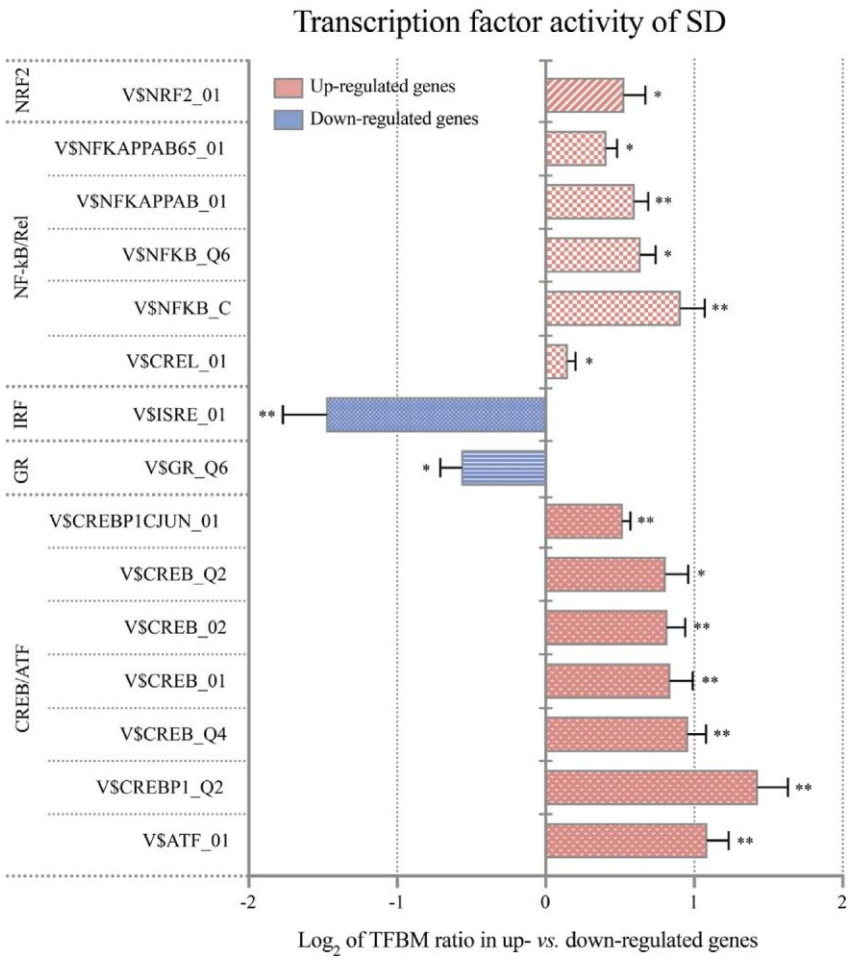


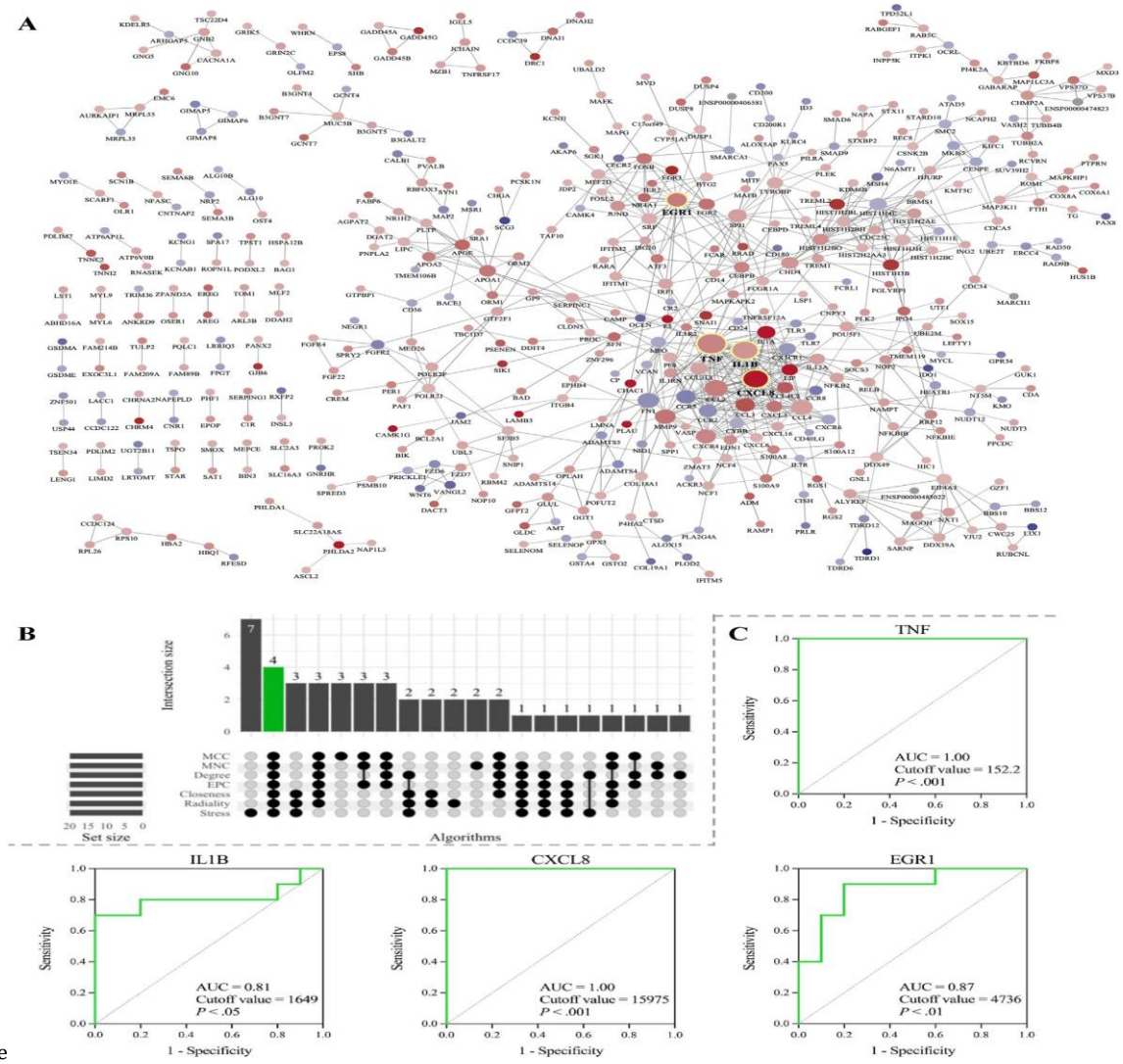
Fig. 3. Magnitude and direction of transcription factor (CREB/ATF family, NF-kB/Rel family, IRF family, GR, and Nrf2) activity in the SD group compared with those in the healthy control (HC) group.
Notes: NRF2: nuclear factor erythroid 2-related factor 2; NF-kB/Rel: nuclear factor kappa B/Rel proto-oncogene; IRF: interferon regulator factor; GR: glucocorticoid receptor; CREB/ATF: cyclic AMP response element-binding/activating transcription factor; TFBM: gene prompter transcription factor-binding motif. The length of the stripe represents $\log_2$ ratios averaged over nine combinations of promoter length and TFBM detection stringency. Error bars indicate standard errors. Positive (red) and negative (blue) values indicate increased and decreased activity of the transcription factors, respectively. Error bars indicate standard errors. $^{**}P < .001$, $^{*}P < .01$ *vs.* healthy control group.

irinotecan, have been used in the fields of infectious diseases, oncology, hematologic malignancy, dermatology, and cardiology. Parthenolide, triciribine (BRD-K80431395), ABT-751, and triciribine (BRD-A42649439) are currently undergoing clinical trials, whereas NSC-663284 and devazepide are still in the preclinical study phase. In addition, NSC-663284 (MOA: CDC inhibitor), mebendazole (MOA: tubulin inhibitor, a launched anthelmintic used to treat hookworm, pinworm, and roundworm infestations), ON-01910 (MOA: PLK inhibitor), and BCI-hydrochloride (MOA: protein phos- phatase inhibitor) acted directly on CDC25C, TUBB4B, PLK2, DUSP1, and 4 SD DEGs, encoding the proteins separately (Supplemental Table 10).

2.14. Network pharmacology analysis of the curative mechanisms of Danzhi Xiaoyao powder in subthreshold depression

In TCMSP and TCMID, 134 effective active compounds of DZXY were screened based on criteria including OB ≥ 30%, DL ≥ 0.18, and HL ≥ 4. In addition, the verified anti-depressant compounds, including ferulic acid (DL ¼ 0.058), glycyrrhizic acid (OB ¼ 19.61, DL ¼ 0.11), geniposide (OB ¼ 14.64), daidzein (OB ¼ 19.44), atrac- tylenolide III (DL ¼ 0.17), hesperidin (OB ¼ 13.33), naringin (OB ¼ 6.92), puerarin (OB ¼ 24.03), and meranzin hydrate (DL ¼ 0.17), of DZXY were supplemented according to the literature. Thus, we identified 143 active compounds of DZXY, the details of which are listed in Supplemental Table 11. Following the removal of targets with a reliability score of 0 and duplicates, 310 potential targets of DZXY active components were obtained. The corre- sponding genes of the 310 target proteins were searched in the UniProt database and detailed information is shown in Supplemental Table 12. After inputting the DZXY active compounds and potential targets into Cytoscape, the DZXY "Compounds-Tar- gets (C-T)" network (Fig. 6A) was created. This network contained 434 nodes and 2090 edges. The network analysis tool revealed a network centralization of 0.335. Network heterogeneity was 1.648 and the average closeness centrality was 0.317, indicating that some nodes in the network were more concentrated than others and contributed more. The average degree of the network was 9.631, with 77 component nodes and 40 target nodes above this value. Node E1 (quercetin) had the highest degree value, being connected to 154 target nodes.

The Venn diagram and heat map (Fig. 6B and C) displayed 30 common targets (DT) of SD and DZXY active compounds, including IRF1, TNF, IL1A, NFKBIA, and F3, and their differential expression between patients with SD and the healthy controls. These genes may play vital roles in the curative effects of DZXY in SD and



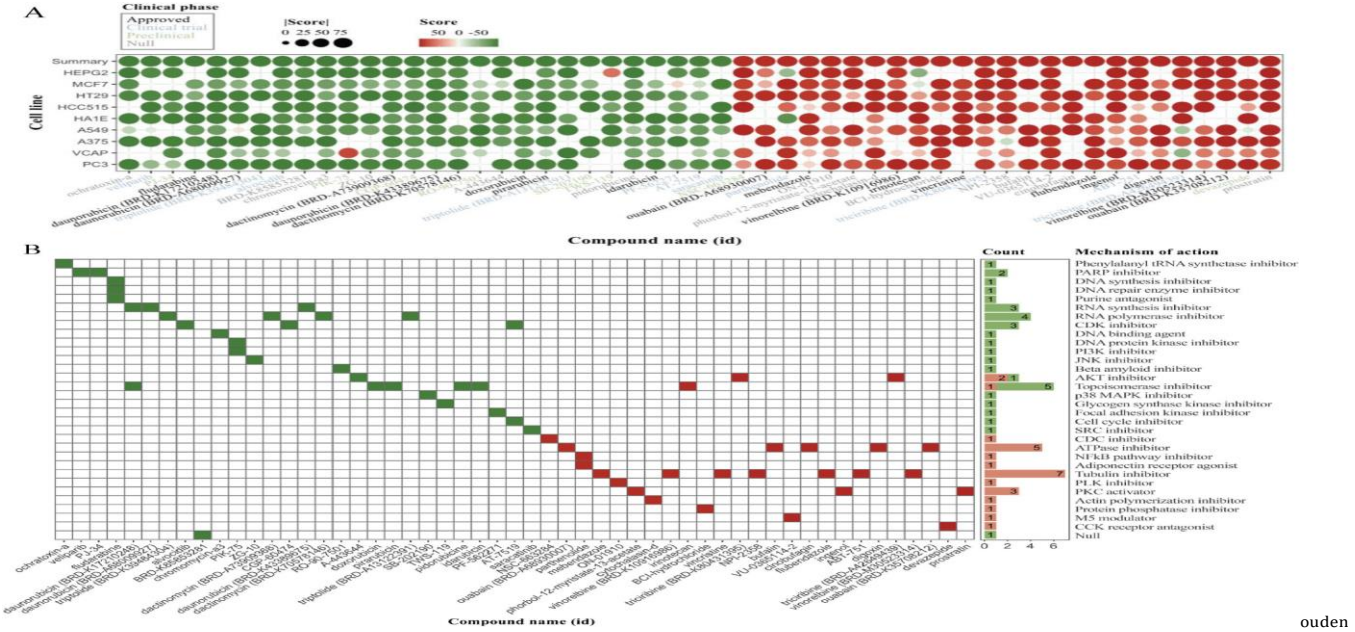


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Fig. 4. PPI network of SD DEGs, SD-related hub gene identification, and ROC curve analysis: PPI network of SD DEGs (A); the UpSet plot displays 4 overlapping hub genes identified from 7 groups (B); ROC curve analysis revealed the predictive performance of hub genes for SD (C).

Notes: SD: patients with subthreshold depression; HC: healthy control subjects; MCC: maximal clique centrality; MNC: maximum neighborhood component; EPC: edge percolated component. In A, the circle node indicates protein encoded by a DEG, filled red color indicates that the gene is upregulated in the SD group compared to that in the healthy control (HC) group. Conversely, the blue filled color indicates that the gene is downregulated in the SD group compared to that in the HC group. The shade of the color reflects the degree of the up/down regulation, and the size of node, from small to large, represents the network connectivity degree. Nodes with a yellow border signify SD hub genes, and the thickness of the gray lines, from thin to thick, indicates the combined score of protein-protein interaction, from low to high (0.7e0.999). In C, the cut-off value corresponded to the gene expression level (fpkm) of the largest Youden index, and the Y ouden index was calculated as sensitivity plus specificity minus 1.

Fig. 5. Connectivity Map analysis exploring potential candidate drugs and pathogenesis for SD: bubble matrix plot displaying Connectivity Scores (tau scores) of SD DEGs to predict candidate compounds in nine cancer cell lines and summary conditions (A); heat map showing the mechanisms of action (rows) of predicted candidate compounds (B).

Notes: In A, the size of the dots corresponds to the absolute values of the Connectivity Scores. The colors of compound names indicate the clinical application or study status of the compounds: approved for use in patients (black), currently or previously in clinical trials (blue), demonstrated efficacy in animal models (green), and currently lack study reports (gray). In B, he compounds with summary Connectivity Scores >95 are displayed with red squares, and those with summary Connectivity Scores < —95 are represented by green squares.

the potential to serve as peripheral immunological indicators for monitoring and evaluating the efficacy of DZXY.

Quercetin, luteolin, puerarin, daidzein, and 19 other active compounds (Table 1) in DZXY targeted the aforementioned 30 DTs. Using the ClueGO and CluePedia plug-ins of Cytoscape, we constructed a "DC-DT-P^{00} three-layer network (Fig. 6D) to depict the properties of the multi-targets and multi-pathways of the multi-active-ingredients of DZXY in SD treatment. Here, the compound quercetin (labelled E1) showed corresponding re- lationships with 19 target genes (degree ¼ 19), and its connec- tivity in the network was much higher than that of other compounds (average degree of compound ¼ 3.16). Thus, it may be a key component of DZXY in SD treatment. The target gene *TNF* (degree ¼ 35) was enriched in 22 pathways (23 in total) and *IL1B* (degree ¼ 29) was enriched in 20 pathways. Thus, they may be the hub genes for the DZXY treatment of SD. Hierarchical clustering analysis divided these enriched annotation items and pathways into eight clusters based on the Kappa coefficient ≥0.6, each represented by distinct categories such as lipids and atheroscle- rosis, the NF-kappa B signaling pathway, rheumatoid arthritis, pertussis, the TNF signaling pathway, osteoclast differentiation, fluid shear stress and atherosclerosis stress, and atherosclerosis. These clusters include inflammatory response, lipid metabolism, immune response, and other processes. Therefore, the curative mechanisms of DZXY in SD may be achieved by the modulation of these pathways.

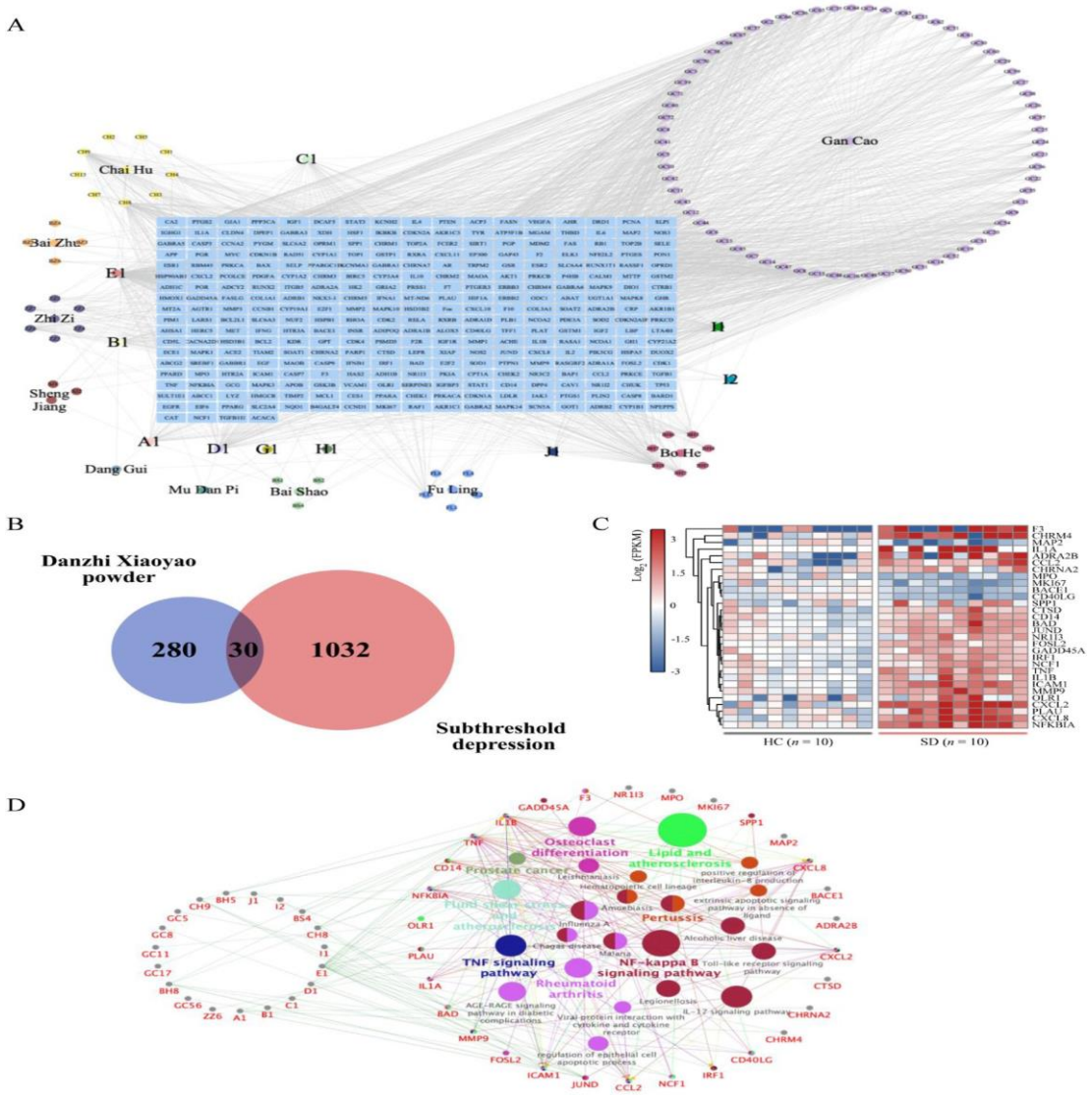

Fig. 6. Network pharmacologyanalysis of the curative mechanisms of Danzhi Xiaoyao powder in subthreshold depression: Danzhi Xiaoyao powder "Active Compound-Target (C-T)" network (A); Venn diagram showing that there are 30 common targets (direct target, DT) of subthreshold depression and DZXYactive compounds (B); heat map displayingthedifferential expression of 30 direct targets between subthreshold depression patients and healthy controls (C); Danzhi Xiaoyao powder "Direct Compounds-Direct Targets-Pathways (DC-DT-P)" network (D). Notes: SD: subthreshold depression; HC: healthy control. In A, the circular nodes of different colors represent the active compounds of DZXY, and the blue rectangular nodes represent the target genes of theactive compounds. In D, the 19 small gray circular nodes ontheleft represent theactive compounds of DZXY, the 30 smallcircular nodes intherightouter layer represent the direct targets of DZXY, and the colored circular nodes in the right inner layer represent the significantly enriched KEGG pathways/GOBP annotation items. Different colors represent different hierarchical clusters, and the pathways/annotation items and target genes involved in multiple hierarchical clusters are displayed as nodes spliced by multiple colors. The green edges connecting the left and right small nodes indicate correspondence between the active compounds and targets, and the colored edge inside the right pattern indicates that a target gene is enriched in a pathway/annotation item.

Table 1
Direct targets and corresponding active compounds of Danzhi Xiaoyao powder.

Com_ID	Herbal name	Molecule name	MW	OB (%)	DL	HL	Degree
A1	*G. Fructus, P. Radix Alba, Z. Officinale Roscoe, A. Sinensis Radix*	B-sitosterol	414.79	36.91	0.75	5.36	4
B1	*G. Fructus, Z. Officinale Roscoe, B. Radix, A. Sinensis Radix*	Stigmasterol	412.77	43.83	0.76	5.57	1
C1	*G. radix, B. Radix*	Isorhamnetin	316.28	49.60	0.31	14.34	2
D1	*M. Haplocalycis Herba, G. radix*	Naringenin	272.27	59.29	0.21	16.98	1
I2	*G. Fructus, P. Radix Alba, G. radix*	Kaempferol	286.25	41.88	0.24	14.74	4
J1	*M. Haplocalycis Herba, G. radix*	Hesperidin	610.62	13.33	0.67	NA	1
BH5	*M. Haplocalycis Herba, A. Sinensis Radix*	Ferulic acid	194.20	39.56	0.06	2.38	2
E1	*G. Fructus, G. radix, C. Moutan Radicis, B. Radix*	Aloe-Emodin	270.25	83.38	0.24	31.49	2
BH8	*M. Haplocalycis Herba*	Quercetin	302.25	46.43	0.28	14.40	19
BS4	*P. Radix Alba*	Luteolin	286.25	36.16	0.25	15.94	6
CH8	*B. Radix*	Paeoniflorin	480.51	53.87	0.79	13.88	2
CH9	*B. Radix*	Daidzein	254.25	19.44	0.19	NA	5
GC11	*G. radix*	Puerarin	416.41	24.03	0.69	NA	5
GC17	*G. radix*	Euchrenone	406.56	30.29	0.57	15.89	1
GC5	*G. radix*	Naringin	580.59	6.92	0.78	NA	1
GC56	*G. radix*	Medicarpin	270.30	49.22	0.34	8.46	1
GC8	*G. radix*	Licochalcone A	338.43	40.79	0.29	16.20	1
		Formononetin	268.28	69.67	0.21	17.04	1

issue damage and bacterial infection following trauma, posed more hazardous and frequent threats to human life and health. To manage these potential or perceived threats, the "fight-or-flight"- related signaling pathways in the SNS are activated, releasing norepinephrine that acts on the beta-adrenergic receptors of im- mune cells, inducing increased CREB and NF-kB/Rel activity and decreased IRF activity downstream, finally shifting the basal tran- scription status of immune cells towards a proinflammatory state, a response the body perceives as contributing to defense risks.[9,23] In contemporary society, chronic low-intensity social stress is more troubling for certain groups of people, such as those in bereave- ment, those with low socioeconomic status, and those who expe- rienced childhood maltreatment. Similar to previous physical threats, these psychosocial stimuli can activate the SNS and elicit a CTRA pattern (proinflammatory/anti-interferon skewing) tran- scription expression in innate immune cells, leading to a persistent inflammatory state in the body, associated with multiple diseases, including depression.[9,23,24,38e41] Although the HPA axis is also autonomically activated to resist various stressors, the consequent increase in circulating glucocorticoids (GCs) following chronic stress cannot block inflammation because GC resistance/impaired GR activity is established by chronic stimulation. Thus, the reduced anti-inflammatory function of GRs further promotes inflammation.[40,42]

Undeniably, social environmental risk factors (social adversities) play an important role in precipitating mental disorders. A link exists between immune CTRA-mode transcriptional profiles and depression/depressive symptoms.[24,38,43] Antidepressant treatment, cognitive behavioral interventions (a classic psychotherapy rec- ommended for SD and MDD treatment),[7] and some wellness practices, such as yoga, tai chi, and meditation, which are confirmed complementary therapies for depressive disorder,[44e47] downregulate CTRA gene expression profiles under basal condi- tions and in highly threatening conditions, such as childhood low socioeconomic status or cancer diagnosis.[24,48,49] Hence, we sus- pected that the pathogenesis of SD may be relevant to CTRA mechanisms; that is, the immune transcriptional profiles of pa- tients with SD may share some characteristic components of CTRA.

Discussion

Although more than 100 years have passed since SD was first mentioned as a pre-depressive state, and clinical observations and epidemiological studies have noted that MDD and SD share several similarities regarding risk factors, demographic characteristics, and symptoms,[17,36,37] the pathogenesis of SD remains poorly under- stood because of methodological limitations and clinical concerns. Recently, with widespread attention on physical health and the development of high-throughput "omics" technologies, researchers found that different psychosocial risk factors evoked a common transcription profile pattern, known as CTRA, in immune cells (primarily leukocytes) of different species. The pattern is charac- terized by the increased expression of proinflammatory genes and decreased expression of genes involved in innate antiviral re- sponses and antibody synthesis.[9,10,23,24] From the evolutionary theory perspective, CTRA is analogous to a defensive program built under ancestral conditions, when acute and transient risks, such asfunctional annotation, and performed KEGG/Reactome pathway enrichment analysis for SD DEGs. Additionally, our focus extended to evaluating the activity variations of four transcription factor control pathways (CREB/ATF family, NF-kB/Rel family, IRF family, GR) in patients with SD compared with healthy controls. Acknowledging the close clinical association between SD and MDD, along with well-established theories and hypotheses of MDD pathogenesis, we also analyzed the activity of the Nrf2 transcrip- tion factor in patients with patients. By combining this analysis with the variations in the activity of the first four transcription factors, we aimed to decode the correlation between SD and MDD in pathological mechanisms simultaneously.[3e7,9,38,50e53]

Despite the relatively fewer symptoms and shorter duration in patients with SD, we still identified significant differences in leukocyte transcriptional profiles between patients with SD and healthy individuals. Subsequently, functional enrichment analyses showed that the DEGs of SD in leukocytes were primarily involved in biological processes related to the cellular response to chemo- kines, cellular responses to biotic stimuli, regulation of inflamma- tory responses, and molecular functions related to intra- and extracellular signal transduction activities, including DNA-binding transcriptional activator activity, G protein-coupled chemo- attractant receptor activity, and chemokine and cytokine activity. "Gene Ontology" functional tagging of CTRA-pattern differentiallyexpressed genes in leukocytes pertained to inflammation, chemo- kine activity, and cytokine activity.[40] The GO cellular component tagging of SD DEGs mainly concerned endocytosis and exocytosis, such as phagocytic vesicles, tertiary granules, specific granules, etc. Among them, phagocytic vesicles arise from the ingestion of par- ticulate material via phagocytosis, which plays an important role in cellular immunity. Monocytes, macrophages, neutrophils, etc., are professional phagocytes, primarily responsible for eliminating mi- croorganisms and presenting them to cells of the adaptive immune system.[54] Tertiary and specific granules were primarily found in mature neutrophil cells and are involved in regulating the innate immune response and promoting the inflammatory process.[55] Furthermore, the SD DEGs were implicated in KEGG pathways, including signal transduction, infectious diseases, autoimmune disease, phagosome, and cell death. Notably, the nuclear factor kappa-B (NF-kB) signaling pathway was the most
significant KEGG enrichment pathway (P ¼ 2.63E-07, rich
factor ¼ 0.202). The NF-kB /Rel transcription factor family plays a role in mediating immunity and inflammation,[56] and the activa-
tion of the proinflammatory transcription factor NF-kB-controlled pathways is a core characteristic of CTRA.[9,23] The overexpression and overactivation phenomena of the NF-kB

regulatory pathway were also observed in the peripheral blood and central nervous system of patients with depression and related animal models.[43,57e59] The enriched Reactome pathways for the DEGs included NGF-stimulated transcription, interleukin-10 signaling, chemokine receptors binding chemokines, diseases of the immune system, ROS and RNS production in phagocytes, etc. These pathways were classified into two categories. One is represented by NGF-stimulated transcription, which includes four pathways, signaling by NTRK1 (TRKA), signaling by NTRKs, and nuclear events (kinase and transcription factor activation). NGF binds to its receptor, neurotrophic receptor tyrosine kinase 1 (NTRK1), and activates phosphatidylinositol-3 kinase (Pl3K) and mitogen-activated protein kinase (MAPK), consequently leading to phosphorylation of CREB, a transcription factor that translocates into the nucleus, controlling the expression of anti-apoptotic genes. Based on the above mechanisms, NGF regulates the survival of immune cells and neurons.[60,61] Several neurodegenerative diseases, including depression, have abnormalities in the function/levels of NGF and its receptors.[50,62e64] Furthermore, through its effects on neuronal and immune cells, NGF can directly or indirectly regulate the peripheral immune response and inflammation.[61,65] The other category is closely related to the immune system and their diseases. Notably, ROS and RNS production in phagocytes is one of the host defense microbicidal events in the innate immune system and serves as an important player in antigen presentation for ensuing adaptive immune responses; however, if this process is out of control, the excessive production of ROS and RNS would lead to oxidative stress.[66,67] Redox homeostasis was often aberrant in patients with depression, resulting in enhanced oxidative stress and impaired antioxidant defense responses.[52,68,69] In addition, ROS were implicated in promoting the release of inflammatory mediators.[52] The function and pathway enrichment results of SD DEGs indicated that immune dysfunction, inflammation and potential pro-/antioxidant imbalance may be part of the pathological process of SD.

We analyzed variations in the activity of upstream transcriptional control pathways between patients with SD and healthy participants, hypothesized to be associated with the biological themes of CTRA and to be activated in association with MDD.[9,23,24,43] Aligned with the specific functional themes of the CTRA molecular profile, both the cyclic AMP response element binding protein/activating transcription factor (CREB/ATF) TF family and pro-inflammatory NF-κB/Rel TF family showed significantupregulation activity in the leukocyte transcriptome of patients with SD. The CREB/ATF TF family, which is ubiquitously expressed, participates in regulating cell proliferation, adaptation, survival, differentiation, and other cellular processes by controlling target gene expression in response to up to 300 different types of stimuli, including peptide hormone stimulation, growth factor stimulation, and neuronal activity.[70e72] In neural cells, the target genes of activated CREB/ATF TFs, such as c-Fos, leptin, BDNF, and miR132, have been shown to profoundly contribute to neuronal circuit development, existing neuron survival, and neurite outgrowth. These findings suggested that CREB/ATF TFs function as neuroprotectants.[71] In addition, experimental evidence clearly suggested that CREB/ATF dysfunction is associated with the pathogenesis of several neurodegenerative diseases and mood disorders (including MDD).[71,73e75] In the immune system, CREB/ATF activation, regulating the NF-κB/Rel controlling pathways, indirectly regulates the expression of TNF, interleukin-2, interleukin-10, and chemokine ligands, playing a role in the dual-directional regulation of the inflammatory response.[71] Increased CREB/ATF activity and/or expression in peripheral blood mononuclear cells, lymphocytes, and leukocytes has been observed in depressive symptom-appearing individuals and animal models of depression.[24,43,74,76] In addition, we observed a significant overrepresentation of IRF and GR TFBMs among downregulated promoters in the leukocyte transcriptome of patients with SD compared to healthy controls. IRF family TFs, which mediate signaling via type I interferons, are involved in antiviral infections and immune modulation.

Mechanistic studies on animal and cell culture systems have shown that the β-adrenergic receptors mediating SNS activation of CTRA upregulate proinflammatory gene transcription, simultaneously inhibiting the activity of TFs that control the transcription of type I interferon genes, such as the IRF family.[23] In addition, with persistent adverse social condition stimulation, β-adrenergic signaling-mediated SNS activation promotes the production of immature, proinflammatory monocytes by altering hematopoietic processes, and the upregulated *NGF* supports the growth and differentiation of the SNS nerve fibers innervating lymph nodes, which further promotes CTRA transcriptome shifts of increased inflammatory responses and decreased antiviral immune responses.[10,23,77] The attenuation of IFN-I signaling was also observed in patients with MDD and related model mice, and this decreased IFN-I antiviral activity was confirmed to be associated with elevated arginine vasopressin from the HPA axis.[78] The treatment-associated upregulation of IFN-I upstream regulatory and downstream targeted transcripts was observed in antidepressant-treated patients with MDD.[24,79] Contrary evidence still indicates increased IFN-I signaling in the peripheral blood of depressed individuals. However, pharmacologic type I interferon, especially IFN-α, treatment induces depressive symptoms in hepatitis C, malignancies, and patients with multiple sclerosis.[80e82] Researchers suspected that IFN-I activated the enzyme indoleamine 2,3-dioxygenase, promoting peripheral and CNS tryptophan depletion, ultimately leading to serotonin reduction. This may explain the effects of increased IFN-I in depression pathogenesis, although the precise cause of the upregulated IFN-I signaling remains ambiguous.[82e84] Overall, the dysregulated innate antiviral immune system plays a role in the pathogenesis of depression. Both the themes of CTRA and the mechanisms of MDD involve a diminished HPA axis negative feedback regulation circuit that is mediated by the downregulation or decreased expression of *NR3C1*, a GR-encoding gene.[38,42,43,49,85,86] GR is a glucocorticoid-triggered nuclear transcription factor ubiquitously expressed in multiple tissues. Activated GR plays a role in anti-inflammatory actions, neurogenesis, and the regulation of glucose and fat metabolism, relying on direct binding to the regulatory regions of its target genes, termed "transactivation".[42,87] In addition, the immune-regulating effects and neurotrophin expression regulation of activated GRs are also implemented by "transrepression", in which GR functions as a monomer binding to other transcription factors, such as NK-κB and CREB, and inhibiting target gene expression.[42,87] Thus, attenuated GR biofunctions may play a role in the mechanisms of CTRA and the etiopathogenesis of MDD and SD in two main ways.

The promoter TFBM enrichment analyses results showed the significant overrepresentation of response elements for the antioxidant TF Nrf2 (V$NRF2_01) in the promoters of genes upregulated in association with SD. NRF2 is a ubiquitously expressed transcription factor that functions as a redox homeostasis-sustaining master, a regulator of metabolism and mitochondrial function, and a maintainer of proteostasis, although at different levels in different cell types. It also participates in regulating cellular autophagy and inflammatory and immune responses.[52,53,88,89] Oxidative stress is an accepted potential mechanism of MDD that initiates or aggravates a series of depression development-related pathophysiological processes, including mitochondrial dysfunction, neuroinflammation, autophagy disorder, and ferroptosis.[24,90e93] Various depression models and *in vitro* experiments have shown that irregular Nrf2 activity/expression is accompanied by oxidative stress in depression. Both antidepressants and antioxidants, such as sertraline, fluoxetine, edaravone, and Mito-TEMPO, ameliorate depressive behaviors and restore

redox homeostasis by normalizing Nrf2-mediated signaling pathways.[24,52,90,94] Furthermore, although CTRA does not address redox homeostasis, increased activity of Nrf2 was discovered in a mechanistic study related to the proinflammatory skew of leukocyte CTRA. In this context, Nrf2 was regarded as a proinflammatory TF only, representing one aspect of the inflammatory features of CTRA.[95] However, Nrf2 activation can either suppress or promote the inflammatory response, antiviral immune response, and anti-tumor immunity in a cell type- and disease context-dependent manner.[88] Thus, it is premature to definitively the role of NRF2 activation in CTRA without a thorough investigation into the complicated mechanisms involved. Overall, the TF activity analysis results indicated that the leukocyte transcriptional profiles of patients with SD shared some common features with CTRA in the immune system and pathogenesis of patients/animal models of MDD.

To search for potential diagnostic indicators and therapeutic targets of SD, we constructed a PPI network of SD DEGs and identified four hub genes, *TNF*, *IL1B*, *CXCL8*, and *EGR1*, for SD using seven well-accepted algorithms (MCC, MNC, Degree, Closeness, Radiality, Stress, and EPC). ROC curve analysis showed that the four hub genes displayed favorable predictive performance for SD (all AUCs >0.7), indicating their potential in the clinical diagnosis and treatment of SD. TNF-, IL1B-, and CXCL8-encoding proteins are important cytokines that play essential roles in the inflammatory and immune responses. The 53 CTRA indicator genes include *TNF*, *IL1B*, and *CXCL8* (also known as IL-8), and these three, and 16 other proinflammatory genes, compose a priori-defined positive indicator set of the CTRA profile.[40,96] In addition, TNF is a well-studied inflammatory factor in the pathological mechanisms of depression, which perturbs the mental health of patients by inducing glucocorticoid resistance and promoting abnormal activation of the HPA axis, affecting neurotransmitter transmission and other processes.[8,97,98] The increased basal level of TNF in depressed patients was negatively correlated with the efficacy of antidepressants in depression treatment, and TNF inhibitors demonstrate the potential to ameliorate the clinical symptoms in certain depressed patients.[99e101] IL-1β, similar to TNF, is generally upregulated in MDD patients, and elevated IL-1β levels are positively associated with the susceptibility and severity of depression.[8,99,102,103] Antidepressant therapy could effectively reduce IL-1β levels in some depressed patients.[8,104,105] The encoded protein of CXCL8, called interleukin-8 (IL-8), is a member of the CXC chemokine family. Clinical trials and animal experiments show a correlation between abnormal IL-8 levels and depression severity and anti-depressant treatment efficacy, and this correlation varies according to sex (it is more common in females), age, and race.[102,106,107]

EGR1, first identified as nerve growth factor-induced protein A (NGFI-A), is a member of the EGR family of early response transcription factors and participates in neuronal differentiation, transcriptional regulation, and other cellular biological processes, such as the regulation of cell proliferation, apoptosis, immune response, adhesion, and inflammation.[108,109] Given the strong association between EGR1 expression and neuronal plasticity, the observed downregulation of EGR1 expression in the hippocampus or prefrontal cortex of depressed patients and stress-induced depression animal models has been identified early and used as a monitoring indicator for investigating the pathogenesis of depression.[109e113] Although EGR1 participates in monocytic and macrophagic differentiation and plays a role in modulating inflammatory responses in mature myeloid cells, EGR1 overexpression in mature cells shows anti-inflammatory effects by reducing cytokine secretion to blunt macrophage activation.[114] The majority of research exploring the functions and expression variations of EGR1 in mood disorders, including depression, has primarily concentrated on its role in the nervous system. There is a lack of concern regarding variations in EGR1 expression and biofunctions in the immune systems of patients with depression. In addition, increased EGR1 expression was observed in the leukocyte transcriptome profiles of individuals who experienced adverse life circumstances such as subjective social isolation and low socioeconomic status. These adverse conditions may trigger higher risks, higher severity, and poorer outcomes in multiple diseases by perturbing peripheral CTRA activity. In this context, monitoring EGR1 expression or activity serves as an indicator of early myeloid lineage differentiation regulation.[43,95] The precise implications, effects, and mechanisms of EGR1 expression or activity in CTRA transcriptional alterations require further exploration in future studies.

In this study, we identified 28 potential small-molecule compounds capable of reversing the altered expression of the top 400 significant DEGs and ameliorating SD. Additionally, CMap analysis revealed 25 small molecules that showed high similarity with patients with SD in the transcriptome profiles of the top 400 significant SD-specific DEGs. Among the 28 compounds, SB-202190 (summary Connectivity Score = —97.08) is a type of p38 MAPK inhibitor, and there have been several attempts to apply various p38 MAPK inhibitors in the clinical treatment of proinflammatory diseases.[115] Other p38 MAPK-specific inhibitors, such as PD-169316 or SB-203580, exhibit high structural and specificity similarity, with SB-202190 serving as a substitute for SB-203580 in experiments.[116] These inhibitors demonstrated the capacity to reduce serotonin (5-HT) uptake in synaptosomes by decreasing the expression and activity of serotonin transporter (SERT).[117] SERT, mediating high-affinity reuptake of 5-HT in neuronal and non-neuronal cells, is the primary target for the most widely prescribed antidepressant drugs, i.e., selective serotonin reuptake inhibitors, such as fluoxetine, paroxetine and citalopram.[118,119] Proinflammatory cytokines, such as TNF-α and IL-1β, increased SERT activity *in vitro*. SB203580 attenuates the proinflammatory cytokine-mediated stimulation of 5-HT transport to some extent.[118e120] Given the demonstrated anti-inflammatory effects of p38 MAPK inhibitors and their ability of restrain SERT activity, including SB-202190, we hypothesize their potential in the treatment of MDD and SD. However, detailed curative effects and anti-depressant mechanisms require further *in vitro* and *in vivo* Another predicated small molecular component, TWS-119 (4,6-disubstituted pyridopyrimidine, summary Connectivity Score = —97.04), is a specific inhibitor of glycogen synthase kinase (GSK)-3β. GSK-3 (including GSK-3α and GSK-3β two isoforms) is a protein kinase with numerous functions, particularly in neurons, which tightly correspond to neural development, neurogenesis, synaptic plasticity, cell survival and cell death, and neuroinflammation.[121e125] Here, the neuroinflammation-modulating function of GSK-3β partially depends on the regulation of NF-κB and CREB activity, whereas Nrf2, the antioxidative stress and inflammation-resistant TF signaling pathway, is also regulated by GSK-3β.[125,126] With various pharmacological and molecular approaches manipulating GSK-3β activity in animals, deactivation of GSK-3β confers resistance to the appearance of anxious-depressive behaviors.[121,123] GSK-3β polymorphisms and dysfunction of GSK-3β are associated with age of onset, pathological manifestation, severity, and therapeutic responses in patients with MDD.[76,123,126] The mood stabilizer lithium, the rapid antidepressant ketamine, and the classic antidepressants fluoxetine and venlafaxine reverse irregular GSK3 activation *in vivo*.[121,123,125,127] Meanwhile, several GSK-3β inhibitors, such as SB216763, L803-mts, and SAR502250, play an antidepressant role in depression animal models.[128e130] Although there is no report investigating the antidepressant ability of TWS-119, its beneficial efficacies in inflammation amelioration, Nrf2 antioxidant signaling reinforcement, blood–brain barrier

(BBB) maintenance, and neuroprotective effects (including cell apoptosis reduction and synaptic protein expression increase) in hypoxia-ischemic tissue injuries, such as ischemic stroke, preterm infant brain injury, hypoxia-induced cardiomyocyte injury, and immune regulation in cancers, have been verified *in vitro* and *in vivo*.[131e137] Considering the revealed immune dysfunction, inflammation, and potential pro-/antioxidant imbalance in patients with SD, and established findings of neuroinflammation, BBB permeability change, and synaptic plasticity deficits confirmed in depression pathogen- esis,[71,138] we speculate that TWS-119 has particular value in the treatment of SD. However, the effects and mechanisms of action of TWS-119 need to be validated through further experimental evi- dence and clinical trials.

Among the 25 components with Connectivity Scores >95, hor-
bol-12-myristate-13-acetate, ingenol, and prostratin shared the ability to activate the protein kinase C (PKC) pathway). Abnormal- ities in the PKC signaling pathway have been reported in animal models of depression, whereas there were opposing voices, anti- or pro-depression, of the efficacies of PKC activators.[139e141] Ouabain
(BRD-A68930007), bufalin, cinobufagin, digoxin, and ouabain (BRD-K35708212) are ATPase inhibitors. Inhibition of Na^+-K^+ ATPase activity is a common pathway in central nervous system disorders, including neuropsychiatric disorders, depression, and
bipolar disorder.[142e146] Ouabain and digoxin, two membrane Na^+- K^+ ATPase inhibitors, have shown definite potential to induce
depression-like behaviors.[143e145] The AKT inhibitor, the machine of action of triciribine (BRD-K80431395) and triciribine (BRD- A42649439), generally act as antagonists in antidepressant medi- cation research.[146,147] Although PKC activation, ATPase inhibition, and AKT inhibition have been confirmed to participate in the pathological processes of depressive disorders, their significance in the diagnosis and treatment of SD and depression remains limited as these mechanisms are involved in several other diseases such as cancers, infections, cardiovascular diseases, and neurodegenerative disease.[68,148e150]

TCM formulas have unique advantages in the treatment of mental disorders owing to their "multi-ingredient, multi-target and multi-pathway" characteristics. According to our network phar- macology analysis results, we partially verified these characteristics in DZXY-treated SD. Of the 143 active ingredients and 310 target proteins of DZXY, we identified 30 potential direct targets of DXZY after SD treatment and their corresponding active compounds. Among the 30 direct target genes, the expression of *MPO*, *MKI67*, *BACE1*, and *CD*40LG in peripheral blood leukocytes of patients with SD was downregulated compared with that in healthy controls, whereas the other 26 genes were upregulated in patients with SD. *MPO*, which encodes myeloperoxidase and primarily expressed in neutrophils, plays an important role in the antibacterial function of neutrophils and mediates local inflammation. *MPO* expression is generally increased in untreated patients with depression,[151e153] which may be involved in the pathological processes of depres- sion by disturbing redox homeostasis.[154,155] By constructing the three-layer "DC-DT-P" network, we found that *TNF*, *IL1B*, and *CXCL8* were the highest connectivity genes, ranking 1, 2, and 5, respec- tively. These three were confirmed as CTRA indicator genes and predicted SD hub genes, and their expression and/or functional abnormalities have been reported in depression-linked research.

Quercetin was the highest connectivity DZXY active compound in the "DC-DT-P" network, existing in 4 herbal medicines, *B. Radix*,
G. radix, *C. Moutan Radicis*, and *G. Fructus*, which interacted with 19 target proteins, including *TNF, IL1B, CXCL8, IRF1*, and *MPO*. Besides the connectivity degree of luteolin (active ingredient of *M. Haplocalycis Herba*, degree = 6), daidzein and puerarin (active compounds of *B. Radix*, degree = 5 each), kaempferol (active
compounds of *B. Radix*, *G. radix*, *C. Moutan Radicis*, *G. Fructus*, and
P. Radix Alba, degree = 4) and b-sitosterol (active compounds of
G. Fructus, *P. Radix Alba*, *Z. Officinale Roscoe*, and *A. Sinensis Radix*, degree = 4) showed above-average connectivity degrees (average degree of compound = 3.16), interacting with multiple target proteins. These compounds may be vital active ingredients of DZXY for the treatment of SD. Except for b-sitosterol (a phytosterol), the
remaining five compounds are flavonoids, a class of compounds widely found in natural plants and considered an important ma- terial basis for the therapeutic effects of Chinese herbal medi- cine.[156] Quercetin has a wide range of pharmacological effects, including anti-inflammatory, antioxidant, anticancer, antibacterial and neuroprotective properties.[157,158] The antidepressant effects of quercetin have been elucidated in multiple animal models of depression, the mechanisms of which involve regulating the levels of acetylcholine and monoamine neurotransmitters, activating the tyrosine kinase receptor B (TrkB)/BDNF signaling pathway to pro- mote hippocampal neuron regeneration, inhibiting the excessive activation of the HPA axis, alleviating inflammatory reactions, resisting oxidation, and inhibiting N-Methyl-D-Aspartate Receptor activity and nitric oxide synthesis to improve synaptic plasti- city.[157e159] Luteolin, daidzein, puerarin, and kaempferol also exhibit antidepressant effects in some cell and animal experiments and share similarities in antidepressant mechanisms with quercetin, including anti-inflammation, anti-oxidation, HPA axis regulation, regulation of neurotransmitters and neurotrophic factors, promo-
tion of neuron regeneration, and inhibition of neuron apoptosis.[160e165] While there is limited research exploring the an- tidepressant effects of b-sitosterol, its antioxidant, anti-inflammatory, immunoregulatory, and hypolipidemic effects have been confirmed in other diseases. Moreover, given the excellent BBB permeability (BBB = 0.986) of b-sitosterol, which allows it to traverse the BBB and directly affect the central nervous cells,
further research on its therapeutic potential in depression is war- ranted. The enriched KEGG pathways and GOBP annotation items of DZXY direct targets, and their hierarchical clusters, indicated that DZXY primarily operates through the regulation of inflammation, immune response, lipid metabolism, and apoptosis in the treat- ment of SD.

The RNA-seq analyses of leukocytes revealed the potential bio- markers and underlying pathogenesis of SD, and partly deciphered
the correlation between SD and MDD in pathophysiology. Com- bined with the CMap database and network pharmacology strate- gies, the medical therapies for SD were also explored, including identifying the candidate small molecular drugs for SD and veri- fying the curative mechanisms of DZXY in SD. However, the study population was not large enough to draw definitive conclusions, and the MDD patient group was not specifically designed, reducing the verification of the pathophysiological correlation between SD and MDD. Hence, further studies involving larger sample sizes and incorporating MDD patient groups are required to strengthen the conclusions of this study. Additionally, the predicated candidate drugs for SD and the curative mechanisms of DZXY in SD should be explored in more detail using animal experiments and clinical trials.

3. Conclusions

Overall, our study demonstrated that SD exhibits a unique expressed genome signature with peripheral blood leukocytes, and that blood cell-derived RNA or proteins, such as *TNF, IL1B, CXCL8*, and *EGR1*, may have significant value for performing

diagnostic functions and identifying disease biomarkers in SD. In addition to the functional annotation and KEGG/Reactome pathway enrich- ment of SD-specific genes, and the leucocyte transcriptome-based TF activity analysis between patients with SD and healthy con- trols, the findings suggest that the proinflammatory and anti- interferon components of CTRA may be associated with the path- ogenesis of SD. Furthermore, the results indicate that, similar to MDD, inflammation overactivation, anti-virus innate immunity inhibition, oxidative stress, and GR dysfunction may be involved in the pathological processes of SD. CMap analysis identified potential small-molecule drugs for SD, including SB-202190 and TWS-119, which directly target SGK1 and JUN (two SD-specific genes), respectively. Finally, using network pharmacology analysis, we explored the active ingredients and curative mechanisms of DZXY in SD. Among the active small molecular compounds, quercetin, luteolin, daidzein, puerarin, kaempferol, b-sitosterol, and others have the potential to directly impact the hub genes of SD (*TNF*, *IL1B*, and *CXCL8*), influencing various pathological processes in SD such as abnormal inflammatory response, apoptosis-related pathways, and irregular lipid metabolism. These results may provide new insight into the characteristic and functional changes of leukocytes in SD, along with potential leukocyte biomarkers for future appli- cations in identifying and treating patients with SD. Meanwhile, the identified small molecular drugs for SD and the mechanism analyses of DZXY in the treatment of SD could aid in providing effective therapies for clinical SD.

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