Bioinformatics-based Prediction of the mechanism and targets for BushenjieduDecoction in preventing relapse of acute leukemia

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1. Abstract

1.1 Background

Leukemia is a lethal myeloproliferative disorder, its' relapse following chemotherapy is the major concern in clinical practice. For a long time, we found that traditional Chinese medicines such as Bushenjiedudecoction (BSJD) have significant effects on delaying relapse. However, the underlying mechanisms are not clear, which limits the clinical application of BSJD decoction.

1.2 Methods

Therefore, we tried to make some explorations in this study. We isolated mesenchymal stem cells (MSC) after treated them with BSJD for proteomic analysis. And then 109 targets were screened out through analysis of the shared proteins of that affected by BSJD and those related to leukemia. Subsequently, the data were analyzed by GO functions, KEGG pathways, PPI network and topological analysis, and then some nodes were selected for animal experiment.

1.3 Results

As a result, we demonstrated the effective targets of BSJD on MSC through bioinformatics analysis and explored the potential mechanism of BSJD from its influence on niches. These targets contains Hspb1、Dnmt1、Mmp2、Thbs1、Crebbp、Hmgb1、Acta2、Cdkn1b、Atg7、Tsc2 and Icam1. Afterwards, we confirmed BSJD reduced the gene expression of ICAM-1 through cultured MSC in vitro.

1.4 Conclusions

We screened the potential targets of BSJD on MSC through proteomics and

bioinformatics analysis, and selected some genes for experimental verification. These studies demonstrated the effect of BSJD on MSC. We hope that this research method could provide a new way of systematically studying the effects of traditional Chinese medicine on diseases.

Key words:

Drug target prediction, gene interaction network, acute leukemia, relapse, predictors.

2. Background

Chemotherapy is the primary choice of adult acute leukemia in clinic, but usually the prognosis is not satisfied (1, 2). Relapse after chemotherapy is the major concern for acute leukemia treatment. Both acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) have low rates of long time remission, especially the response rate is only 30%-40% in ALL (3, 4).AL is a myeloproliferative disease that characterized by malignant clone of hematopoietic stem cells in transformed niches. Bone marrow microenvironment(niche)supports the homing to bone marrow, self-renewal, proliferation and differentiation of hematopoietic stem cells in bone marrows(5). It is consisted of osteocytes, perivascular cells, endodontic cells, adipocytes, macrophages, and mesenchymal stem cells (MSCs)(6). And MSC plays an important role in these components. It is more than a material basis for niche, because it also mediates the homing of HSC through Nest in.(7). Recent studies suggested that leukemia stem cells (LSCs) can transform niche into a LSC growth-permissive and HSC growth-restrictive microenvironment through remodeling activity in the course of leukemia (8). Different cytokines achieves this transformation through variety pathways and thereby mediates LSC mobilization, homing and drug resistance, which eventually become an important cause of clinical recurrence (9). Treatment to hijacked or transformed niches may provide novel therapies to delay the leukemia relapse(10).

As an important part of Chinese health system, traditional Chinese medicine (TCM) has been used extensively. In disease free interval, we use a variety of TCMs as adjuvant treatments to delay the recurrence. In these Chinese patent medicines we found that Bushenjiedudecoction (BSJD) has a more obvious efficacy, but the specific mechanism is still unclear. Although the research of TCM is facing challenges, the developments of new technologies provide new methods to study the complex mechanism of it. Especially, with the emerging of high-throughput sequencing technology, it makes it possible to obtain large-scale protein data and make comparative analysis of proteomic in a single experiment (11, 12).As a

next-generation of high-throughput technology through relative and absolute quantitative proteomics analysis with high sensitivity and repeatability, iTRAQ has been widely used in cancer research (13, 14). Meanwhile, various bioinformatics databases based on the proteomics also provide powerful support for functional annotation of proteins, classifying protein sequences into families, and comparative protein structure modeling (15). They all promote the research on the mechanism and target location of drugs (16). At the same time, it also provides a new objective way of scientific research and evaluation of TCM compounds (17). Inspired by these methods, we designed our experiment as shown in the figure, including: in vivo administration, cells culture, high-throughput sequencing, etc. Through these processes, possible targets of BSJD were obtained and bio-informatics analysis was carried out on these targets. (Fig.1)

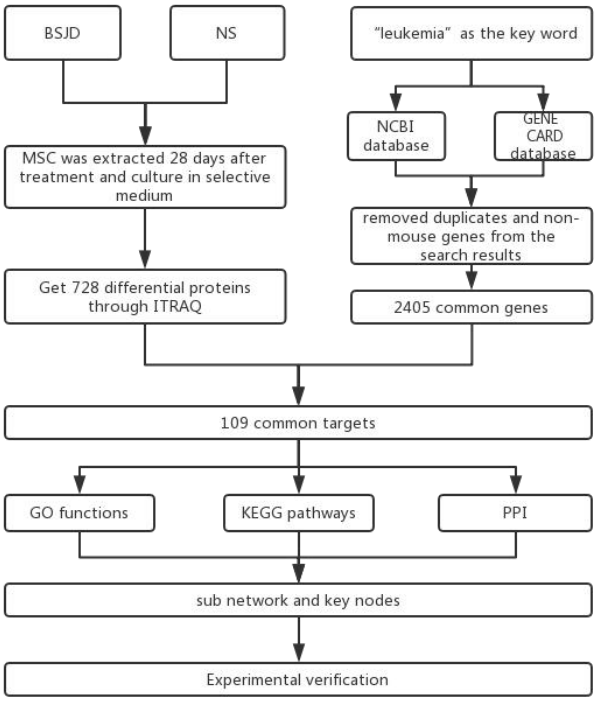


Fig.1|In the flowchart of target prediction and verification, the potential targets of BSJD were screened by proteomics and enrichment analysis, and the functional and net-work of these potential targets were analyzed by GO, KEGG, PPI and topological analysis. Finally, the effect of BSJD on the selected potential targets was demonstrated by experiments.

3. Materials and methods

3.1 Chinese medicine ingredients of BSJD

Bushenjiedudecoction is composed of Quanxie (ButhusmartensiiKarsch), Jiangcan (Bombyx Batryticatus), Baifuzi (TyphoniiRhizoma), Lujiaopian (Cornu Cervi), Guiban (Tortoise Shell Caraoax et PlastrumTestudinis), Renshen (Panax Ginseng C. A. Mey), Gouqizi(Lycii Fructus),Gancao(licorice),Qingdai (Indigo

Naturalis), Huanshi (Talc Powder PulvisTalci).

3.2 Proteomics

The high-throughput sequencing was identified by Tianjin Seweisi Biotechnology Co. Ltd. through ITRAQ technology developed by ABSCIEX （Pro.No.QLSWS003-20190614001）.

3.3 Prediction of common targets for BSJD and leukemia

Leukemia as the key word was searched in two data bases: NCBI (https://www.ncbi.nlm.nih.gov/gene/)and GENE CARD(https://www.genecards.org/).We removed duplicates and non-mouse genes from the search results and found 2045 common targets in two databases. Subsequently, these differential protei ns were compared with 728 differential proteins after BSJD treatment.

3.4 Analysis of GO, KEGG and key sub net.

GO function and KEGG pathway common targets were analyzed with Uniprot(https://www.uniprot.org/).Co-expression network of common targets was established by Gene Mania (http://pages.genemania.org/). 82 common targets which were greater than medium-confidence （＞0.6） were selected for further analysis through STRING （https://string-db.org/）.In addition, topology analysis was conducted by MCODE to calculate the degree centrality and extract sub-network, in order to identify key nodes. Ultimately, targets of the sub-network were selected to perform further animal experiment.

3.5 Animal experiment

All animal experiments were conducted in the animal center of the Institute of Radiation Medicine Chinese Academy of Medical Sciences （IRM） and they all have been approved. The BALB/c mice were purchased from Beijing Huafukang Biotechnology Co. Ltd. （animal license no. SCXK -20190008）.

7 to 8 week-old male BALB/c mice were used for proteome analysis. Mice were intragastric administrated of 0.2ml BSJD once a day for 4weeks and then got euthanized.

The animal model of ALL was generated using 6-week-old male BALB/c mice. $5*10^5$ L1210 cells were transplanted into half lethally（5Gy, ^{137}Cs γ ra y source, Gammacell-40, Canada） irradiated mice by tail vein injection. And on the 7th day after transplantation, one mouse was randomly selected from each group to evaluate the proliferation of B-ALL cells in the bone marrow by bone marrow smear.

3.6 Treatment protocol

Their chemotherapy regimen was cytoxan（3mg/m^2， for onetime）, vindesine （600mg/m^2 for one time） and prednisone（9mg/kg/day, for 3d）at 10^{th} day after transplanted.

BSJD was prepared as decoction at a concentration of 10g/50ml, oral gavage

treated once a day at 0.2ml.

3.7 Cell Line and Cell Culture

L1210 cells were purchased from the cell compartment of the Chinese Academy of Medical Sciences (Shanghai, China) and cultured in DMEM (GIBCO, #12800017) with 10% FBS (GIBCO，10270106) and 1% penicillin-streptomycin at 37℃ and 5% CO_2.

MSC were obtained from mouse femurs and cultured in MesenCult （STEMCELL, CAT#05513）, the selective medium for mouse MSC. The adherent culture method was used to culture the MSC, and three subcultures were performed before the experiment.

3.8 RNA Isolation and Real-Time PCR Analysis

Total RNA was extracted using TRIzol. Reverse transcription and real-time quantitative PCR was conducted by Real-time PCR Fluorescent Quantitative Kit (ZSTC,ZS-M-1009)on PCR machine (Eppendorf).Target gene primer seq uences:ICAM-1:Forward primer-CTGAGCCTGCTGGATGAGAC, Reverse prim er-GCCACCATCCTGTTCTGTGA; SHP2: Forward primer-ACTGTGCAGACC CTACCTCT, Reverse primer-GCACGGAGAGAACGAAGTCT. The relative e xpression quantity $2^{-\Delta\Delta Ct}$ value was calculated to compare the differences am ong two groups (three technical replicates were used for each gene).

3.9 StatisticalAnalysis

All data were represented in mean ± SD. Statistical analysis was performed using GraphPad Prism 8 software with student's t test ($P < 0.05$ was considered with statistical significance). Histogram was made using the Graphpad software.

4. Results

4.1 Potential Targets of BSJD.

In order to obtain the BSJD target genes, we performed high-throughput proteomics analysis by iTRAQ. First, the 6-week old mice were divided randomly into two groups (n=6). BSJD and Control groups were treated with decoction of BSJD (0.2ml every day） and normal saline (0.2ml every day) respectively. After 28 days treatment, mice were killed and the BM cells were collected. Then MSC were cultured in selective medium for mouse MSC.（Fig.2） A comparative proteomic analysis was carried out between the two groups after three generations. We finally got 728 differential proteins, including303down-regulations and 425up-regulations.

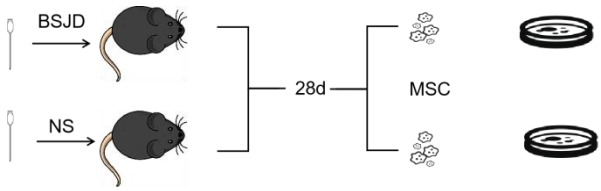


Fig.2| the 6-week-old mice were divided randomly into two groups (n=6). BSJD and Control groups were treated with decoction of BSJD (0.2ml every day） and normal saline (0.2ml every day) respectively. After 28 days treatment, mice were killed and the BM cells were collected.

4.2 Common Targets of BSJD and Leukemia

Being the key word, “Leukemia” was searched in two databases: NCBI and GENE CARD. 20582 relate genes were screened out from NCBI database and10777 were screened out from GENE CARD. When we processed these results, we deleted duplicates and non-mouse genes. To increase the confidence of finally result, we selected 2405 shared genes of both databases for further analysis. At last, we compared these2405 genes with the 728 differentially expressed genes previously identified by proteomics, and then obtained 109 co-expressed genes. These 109co-expressed genes may be the potential targets to reveal the mechanism of BSJD. (Fig.3)

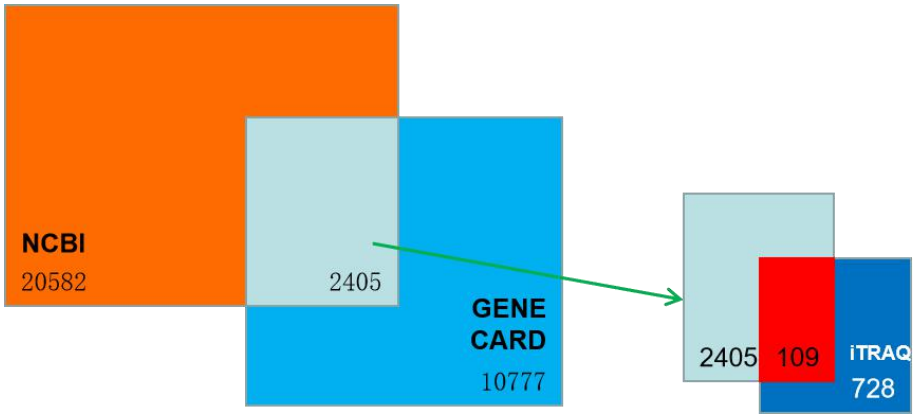


Fig.3| Common targets of BSJD and leukemia

4.3 GO Function and KEGG Pathway Analysis of 109Potential Targets

In order to further analyze these 109 genes, we used the MOU species annotation in Uniprot to annotate their GO functions and KEGG pathways. Uniprot contains millions of real and available protein data, which can be used for protein information analysis of different species (18, 19). This Enrichment analysis can identify valuable functional classes or pathways from identified proteomic results or differentiated protein data (20).The functions were categorized according to their cellular components (CC), molecular functions (MF) and biological processes (BP).（Fig.4, table 1）In the higher ranking results, the words related to the “binding” appeared more frequently.

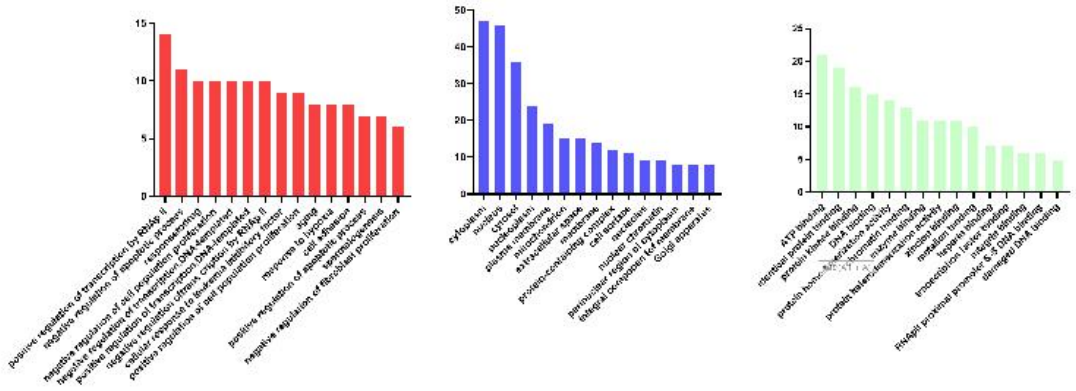


Fig.4| GO enrichment of 109 common genes. Different categories of biological process, cellular component, and molecular function were represented by a red, blue, and green bar.

Tab.1

TermID	Term	gene number	Enrichment	FDR
GO:0019901	protein kinase binding	16	9.907124	0.000000
GO:0005737	cytoplasm	47	9.544287	0.000000
GO:0005829	cytosol	36	8.179951	0.000001
GO:1990830	cellular response to leukemia inhibitory factor	9	8.920293	0.000002
GO:0048147	negative regulation of fibroblast proliferation	6	8.526117	0.000002
GO:0003682	chromatin binding	13	7.560293	0.000004
GO:0042802	identical protein binding	19	7.056140	0.000009
GO:0005634	nucleus	46	6.686909	0.000012
GO:0000790	nuclear chromatin	9	6.811458	0.000012
GO:0042493	response to drug	10	7.443288	0.000015
GO:0019899	enzyme binding	11	6.548856	0.000023
GO:0043200	response to amino acid	5	6.895295	0.000041
GO:0007568	aging	8	6.409238	0.000100

GO:0005654	nucleoplasm	24	5.631881	0.000112
GO:0001666	response to hypoxia	8	6.111689	0.000165
GO:0008201	heparin binding	7	5.260585	0.000353
GO:0042803	protein homodimerization activity	14	4.951369	0.000450
GO:0005178	integrin binding	6	5.075747	0.000451
GO:0003684	damaged DNA binding	5	4.974672	0.000488
GO:0035767	endothelial cell chemotaxis	3	5.452104	0.000644

Tab.1| The top 20 terms of GO analysis.

In addition, the pathway analysis of KEGG is shown in figure 5. We liste d the top 20 pathways, includingmmu01524、mmu05203、mmu01523、mm u05215、mmu00670、mmu05212、mmu05220、mmu04110、mmu05200and mmu05206.Platinum drug resistance, P53 pathway, CML pathway are close ly related to the pathogenesis, treatment and prognosis of leukemia (21-23). Especially in relapsed or refractory leukemia (24), one research showed a significantly increase of platinum-based drugs resistance.(24)

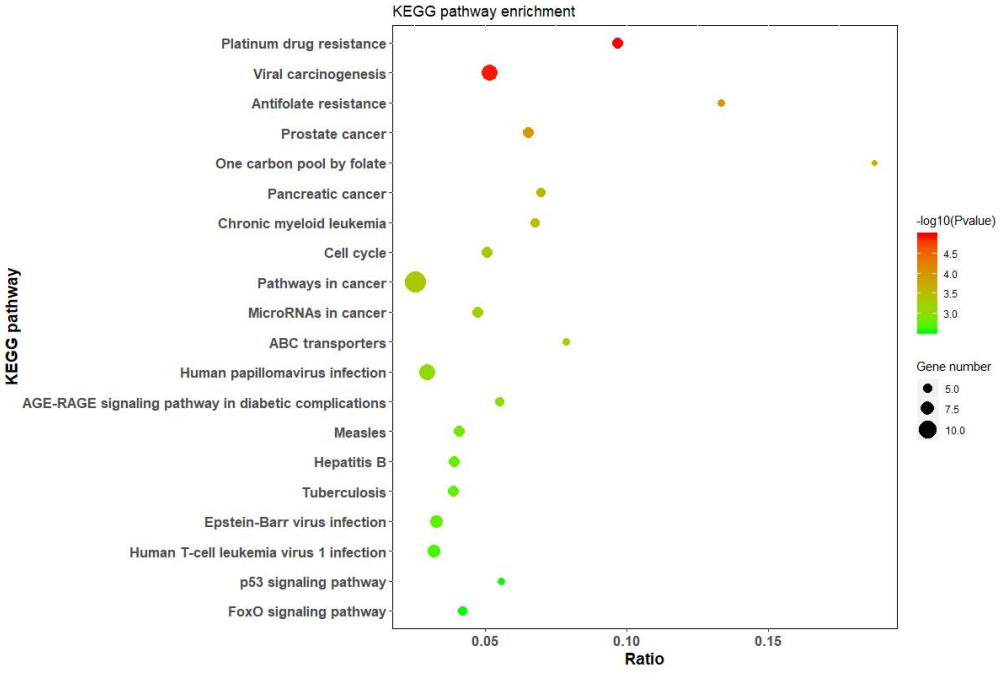


Fig.5| KEGG enrichment of 109 common targets, the size of the bubbles represents the degree of gene enrichment, and the color represents the P value.

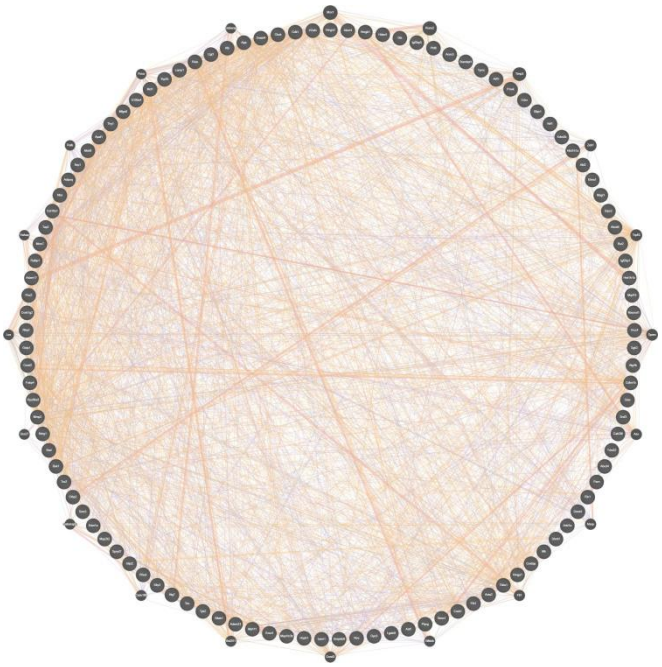
213 Tab.2

Term ID	Term	Term num	Enrichment	FDR
path:mmu01524	Platinum drug resistance	6	4.973571	0.002030
path:mmu05203	Viral carcinogenesis	9	4.935924	0.001107
path:mmu01523	Antifolate resistance	4	4.007099	0.006263
path:mmu05215	Prostate cancer	6	3.992614	0.004857
path:mmu00670	One carbon pool by folate	3	3.555667	0.010627
path:mmu05212	Pancreatic cancer	5	3.525363	0.009496
path:mmu05220	Chronic myeloid leukemia	5	3.469654	0.009253
path:mmu04110	Cell cycle	6	3.380453	0.009942
path:mmu05200	Pathways in cancer	12	3.365681	0.009143
path:mmu05206	MicroRNAs in cancer	6	3.229578	0.011258
path:mmu02010	ABC transporters	4	3.103411	0.013685
path:mmu05165	Human papillomavirus infection	9	3.089588	0.012950
path:mmu04933	AGE-RAGE signaling pathway in diabetic complications	5	3.055819	0.012920
path:mmu05162	Measles	6	2.881639	0.017917
path:mmu05161	Hepatitis B	6	2.792969	0.020510
path:mmu05152	Tuberculosis	6	2.764337	0.020539
path:mmu05169	Epstein-Barr virus infection	7	2.744732	0.020223
path:mmu05166	Human T-cell leukemia virus 1 infection	7	2.665063	0.022946
path:mmu04115	p53 signaling pathway	4	2.544895	0.028667
path:mmu04068	FoxO signaling pathway	5	2.538302	0.027650

214 Tab.1| The top 20 pathways of KEGG analysis.

215 **4.4 Protein-protein Interaction Network (PPI) Analysis**

216 To further reveal the function of these 109 targets during the interaction with
217 other proteins, we constructed a PPI network through the web tool GENE MANIA.
218 Those 109 targets established a co-expression network with other 20 targets in
219 GENE MANIA by PPI analysis methods including Co-expression, physical
220 interactions, pathways, and Co-localization.（Fig.6）



图表 6

4.5 Co-expression and Topology Analysis of over medium-confidence.

Although we obtained the PPI network of these 109 proteins, their underlying connections cannot be directly reflected by this network structure. Therefore, on the basis of GENE MANIA analysis, we selected 82 proteins with score above 0.6 for k-core analysis in Cytoscape3.7.1 using MCODE.K-core is a topological analysis that decomposes the network relation of interaction, and find out the important nodes in the complex network relation structure (25).In this study, we set the parameters of degree>15 and k-core=2 to identify key nodes in the co-expression network (Fig.7). This sub network contain sHspb1、Dnmt1、Mmp2、Thbs1、Crebbp、Hmgb1、Acta2、Cdkn1b、Atg7、Tsc2 and Icam1.

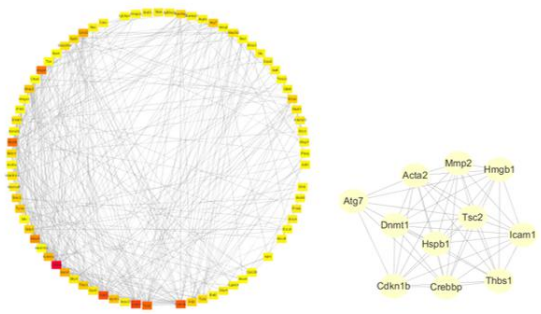


Fig.7| 82 proteins with score above 0.6 for k-core analysis. On the right is the Core sub-network obtained by k-core analysis.

4.6 Sub Network

We've done a literature review of genes' function in this core network, some key genes in the study of leukemia were screened.TheTsc2 gene is involved in PI3K/AKT/mTOR signaling pathways that can be phosphorylated by Akt (26).Tsc2 phosphorylation mediated by different cytokines can promote leukemia cell growth and inhibit apoptotic pathways in both AML and ALL(27, 28). Icam-1 (CD54) is the receptor of lymphocytes cell surface molecule LFA-1, they are both involved in the intercellular adhesion of lymphocytes(29). In leukemia, icam-1 has also been shown to be associated with niches transformation process caused by leukemia stem cells: LSC secrete stimulating factors that enhance the level of Icam-1 in niches, thereby promoting the adhesion between themselves and niches (30). Recent studies demonstrated that Atg7 increased chemo resistance in leukemic cells By combining with EVI1 (31). Moreover, Atg7 plays a key role in the protection of AL cells that was mediated by niche. Inhibiting the expression of Atg7 can enhance the sensitivity to chemotherapy and prolong the survival time of leukemia mice after chemotherapy (32).These evidences suggested that the therapeutic targets of BSJD and leukemia are related at the level of PPI analysis.

4.7 BSJD Reduced the in Vitro Cloning of B-ALL Mouse MSC

In order to verify the targets of BSJD, we constructed an ALL mouse mo de. BALB/c mice were randomly divided into control group and BSJD group (n=6), and L1210 cells were transplanted after 5Gy irradiation（Fig.8a）.Bon e marrow smears confirmed the proliferation of ALL cells in miceafter7days sincetransplantation（Fig.8b）.On the 10^{th} day since transplantation, both two groups were treated with the same chemotherapy of cytoxan, vindesine and p rednisone. In addition, the BSJD treatment group received intragastric admini stration of BSJD decoction from the 7^{th} day since transplantation (0.2ml/day and was lasted three weeks).After 28 days since transplantation, all mice wer e killed and their MSCs were cultured in vitro. As shown in results, through an in vitro colony formation assay, we found that the colony-forming ability of BSJD treated MSC was significantly reduced（Fig.8c）.

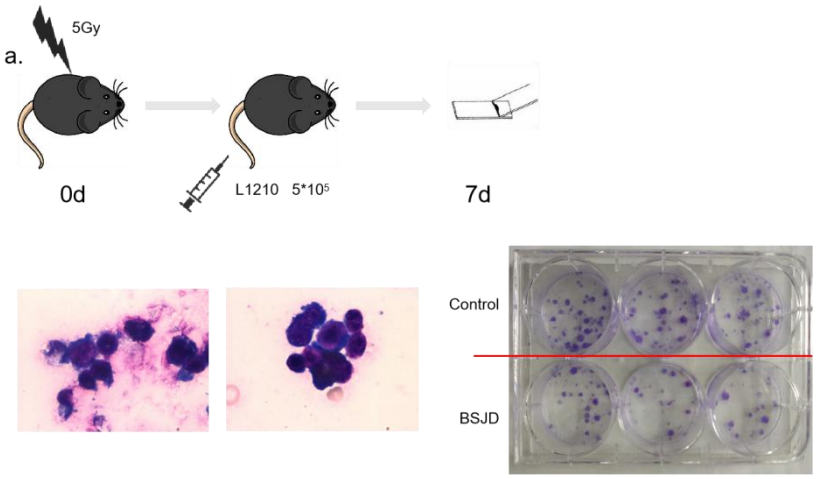


Fig.8| a. The method of ALL mice model; b. At 7th days after transplantation, one randomly selected mouse from each group underwent bone marrow smear, which showed a large proliferation of ALL cells in the marrow; c. The in vitro clonogenic assay of BSJD and control group.

4.8 BSJD down-regulated the expression of ICAM-1 in MSC.

To verify the effect of BSJD on the potential targets, we measured ICAM-1 in BSJD group and control group by quantitative real-time PCR (qRT-PCR).Meanwhile, the downstream targets of icam-1, SHP2, was selected for qRT-PCR detection. Some studies demonstrated that the SHP2 is involved in Scr activation mediated by icam-1 and enhanced adhesion of leukocytes to niche. (33, 34)。 In addition, SHP2 is also involved in the FLT3-ITD-mediated activation of Atg7 to enhance the proliferation and survival of leukemia cells (35). We calculated the mRNA expression values based on the $2^{-\Delta\Delta CT}$ method (36). The results showed BSJD significantly reduced the gene expression of ICAM-1, whereas did not change SHP2 ($P > 0.05$).（Fig.9）。

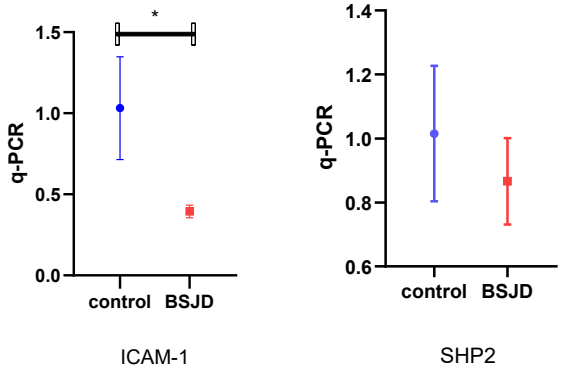


Fig.9|qRT-PCR of BSJD and control group, BSJD significantly reduced the gene expression of ICAM-1.Significance is defined as $p < 0.05$ ($*p < 0.05$).

5. Discussion

LSC transforms niches into tumorous tissues by influencing the stromal cells through growth factors, chemokines, cytokines and anoxic environment in leukemia development process(37). The tumorous niches, in turn, promotes LSC survival, proliferation, and drug resistance through "screening" mechanisms, while crowding out the survival space of normal HSC (38). For example, HIF-1αhigh-expression-mediated microenvironment hypoxia in MSC is one of the important triggers of the niches tumorous process(37). Hypoxia can not only enhance the adhesion and chemotaxis between LSC and MSC, but also reduce the sensitivity of LSC to chemotherapy drugs (39, 40). Down regulated expression of HIF-1αin MSC can improve hypoxia in niches and suppress the activity and invasion of LSC (41). In addition, reversing the hypoxic microenvironment could prolong the survival of leukemia-bearing mice (42). These evidences from one side prove that niches represented by MSC have a vicious cycle with LSC in leukemia. On the one hand, LSC transforms niches into tumor environments through MSC to make them more suitable for survival; On the other hand, timorous niches protect the LSC while crowding out the normal HSC from the bone marrow microenvironment. Targeting the tumor niches may be a potential therapeutic approach for relapsed/refractory leukemia.

Chemotherapeutics for single target is the primary treatment of AL in clinic. Although new drugs keep achieving clinical efficacy, relapse is still the most important obstacle for long-term remission (3, 4). In addition, immunotherapies represented by CAR-T-cell have been proved effective in the treatment of relapse/refractory acute leukemia. But serious and unforeseen side effects and huge medical costs have limited its clinical application(43). Although researchers recognized the interaction between leukemia and tumorous niches, drug developments to reverse tumorous niches, especially tumorous MSC, is still in infancy. Only a small part of the new drug development for AML, such as Uproleselan (gmi-1271), targets the microenvironment (44). In recent years, with the wide application of artemisinin, the research on the effective ingredients in single or compound natural herbs has been paid more attention, which provides a new option for drug research aimed at reversing tumorous niches.

Network pharmacology and biological information analysis provide a new way to study the mechanism of complex components in traditional Chinese medicine. They objectified the synergy of natural drugs or compounds on different targets and pathways (45). However, with no unified database and the differences in search logic between different databases, study of network pharmacology cannot fully reflect the targets of drugs.(46). Therefore, we selected proteomics to comprehensively identify the differential proteins of MSC on BSJD treatment. And then we screened out the targets of BSJD from these differential proteins through bioinformatics analysis. Finally, the potential targets were preliminarily verified by

qRT-PCR. These results demonstrated the effects of BSJD on MSC in leukemia.

We will systematically validate other potential targets in the core sub-network in our following studies. We hope to systematically, comprehensively and objectively explain the regulatory effect of BSJD on MSC after chemotherapy in leukemia, by verifying the interaction between potential targets.

6. Conclusion

We screened the potential targets of BSJD on MSC through proteomics and bioinformatics analysis, and selected some genes for experimental verification. These studies demonstrated the effect of BSJD on MSC. We hope that this research method could provide a new way of systematically studying the effects of traditional Chinese medicine on diseases.

7. List of abbreviations

Abbreviations	**Paraphrase**
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BP	Biological processes
BSJD	Bushenjiedudecoction
CC	Cellular components
LSC/LSCs	Leukemia stem cells
MF	Molecular functions
MSC/MSCs	Mesenchymal stem cells
PPI	Protein-protein Interaction
TCM	Traditional Chinese medicine

8. Declarations

8.1 Ethics approval and consent to participate

Not applicable.

8.2 Consent for publication

Not applicable.

8.3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

8.4 Competing interests

The authors declare no conflicts of interest.

8.5 Funding

This study was supported by the National Natural Science Foundation of China (No.81774048).

8.6 Authors' contributions

WS designed the experiment, performed the major experiments and data analysis; FY designed the experiment and provided technical support, both are major contributor in writing the manuscript. WS, XT, ZX, YL, XX participated in the experiment and organized the literature. All authors read and approved the final manuscript.

8.7 Acknowledgements

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10. Supplementary information

Additional file 1, targets of leukemia in GeneCards.

Additional file 2, targets of leukemia in NCBI.

Additional file 2, con-targets of NCBI and GeneCards.

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