Transcriptome sequencing and metabolome analysis reveal the mechanism of Shuanghua Baihe Tablet in the treatment of oral mucositis

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[ABSTRACT] Oral mucositis (OM) caused by cancer therapy is the most common adverse reaction in the radiotherapy of head and neck tumors. In severe cases, it can lead to the interruption of treatment, which affects the control of the disease and the quality of life. Shuanghua Baihe Tablet (SBT) is a traditional Chinese medicine (TCM) formula, which is administered to treat OM in China. It has been clinically effective for more than 30 years, but the underlying mechanism is not completely understood. With the development of multiple omics, it is possible to explore the mechanism of Chinese herbal compound prescriptions. Based on transcriptomics and metabolomics, we explored the underlying mechanism of SBT in the treatment of OM. An OM model of rats was established by 5-FU induction, and SBT was orally administered at dosages of 0.75 and 3 g·kg⁻¹·d⁻¹. In order to search for SBT targets and related metabolites, the dysregulated genes and metabolites were detected by transcriptomics and metabolomics. Immune related indicators such as interleukin-17 (IL-17) and tumor necrosis factor-α (TNF-α) were detected by ELISA. Treg cell disorders was analyzed by flow cytometry. Our results showed that SBT significantly alleviated the symptoms of OM rats and the inflammatory infiltration of ulcer tissues. After SBT administration, inflammatory related metabolic pathways including linoleic acid metabolism, valine, leucine and isoleucine biosynthesis were significantly altered. Furthermore, the production of proinflammatory factors like IL-17 and TNF-α were also dramatically reduced after SBT administration. Besides, the infiltration degree of Treg cells in the spleen of OM modeling rats was significantly improved by SBT administration, thus maintaining the immune balance of the body. The current study demonstrates that SBT regulates linoleic acid metabolism, glycerophospholipid metabolism and amino acid metabolism, and inhibits IL-17/TNF signal transduction to restore Treg and Th17 cell homeostasis in OM rats, thereby alleviating chemotherapy-induced OM.

[KEY WORDS] Oral mucositis; Shuanghua Baihe tablet; Transcriptomics; Metabolomics; IL-17/TNF signal pathway

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Introduction

Patients with malignant tumors often require radiotherapy and chemotherapy, which however tends to cause a series of adverse effects. Oral mucositis (OM) is one of the most common adverse reactions of cancer therapy. Notably, the incidence of OM in patients with head and neck malignant tumors is relatively high, reaching more than 75% [1]. OM caused by radiotherapy or chemotherapy is an inflammatory response, which is characterized by thinning and damage of oral mucosa, sometimes accompanied by fibrinoporous pseudomembrane overburden and spontaneous bleeding [2]. It can seriously affect the quality of life because of pain, malnutrition and infection. Furthermore, the inability to eat and infection may significantly restrict cancer treatment and reduce
patients' confidence in treatment decisions \cite{1}. Therefore, it is of particular importance to improve the treatment of OM caused by cancer therapy in clinical practice, so as to improve the quality of life.

Traditional Chinese medicine (TCM) has been used in Asia for more than one thousand years. With the discovery and use of TCM monomers such as artemisinin and curcumin, increasing attention has been paid to the research and application of TCM \cite{4,3}. Shuanghua Baihe tablet (SBT) is a TCM prescription used for the treatment of OM over 30 years in China. It contains ten types of medicinal materials, namely Coptidis Rhizoma, C. Bungeanae Herba, Isatidis Radix, Arnebiae Radix, Lonicerae Japonicae Flos, Lophatheri Herba, Rehmanniae Radix, Liliibulbus, Asari Radix et Rhizoma and Snake Bile \cite{6}. SBT has been approved by the National Medical Products Administration (Approval identifier: No. Z20123033) for the treatment of recurrent OM. A double-blind, placebo-controlled study also showed that SBT reduced the occurrence, latency, and severity of OM in patients with nasopharyngeal carcinoma undergoing radiotherapy and chemotherapy \cite{7}. However, there has been no systematic study to explain the underlying mechanism of SBT in the treatment of OM, as traditional Chinese herbal medicine usually works in vivo through multiple components, multiple pathways and multiple targets. As one of the most significant characteristics of TCM compounds in clinical application, synergy determines the necessity of exploring the mechanism of action of TCM compounds from a holistic and systematic perspective \cite{8}.

With the development of high-throughput sequencing, it is increasingly common to use omics as a new strategy to collect biological data. The main advantage of multi-omics joint analysis lies in that these integrated data are collected to analyze the subtle changes in organisms, laying the reliable foundation for the prediction of biological function targets\cite{9}. Combined transcriptome and metabolome analysis is the most common research method in the studies of TCM mechanisms. Transcriptomics can bring a deeper insight into the mechanisms of TCM at the transcriptional or post-transcriptional level. Integrated with transcriptomic data, the results of metabolomic analysis can be more comprehensive and credible \cite{10,11}. Many studies have shown that combined transcriptome and metabolome analysis is an effective approach to evaluating the efficacy of TCM and confirming gene expression, metabolites and metabolic pathways that significantly change after TCM treatment \cite{12}.

In the current study, based on transcriptome and metabolomics, combined with existing molecular biology techniques, we investigated the synergistic effects of the main active components of SBT and explored its potential mechanism. Transcriptome and metabolomics analysis showed that SBT played a vital role in the treatment of OM by affecting immune-related pathways, such as IL-17 signaling pathway, TNF signaling pathway and metabolic pathways like linoleic acid metabolism. These findings proved the rationality of TCM compounds, and indicated that several weak Chinese herbal medicines can be used in combination to exert strong efficacy (Fig. 1).

![Fig. 1 The flow diagram of this study](image-url)
Materials and Methods

Instrument, materials and animals

UHPLC-Q-Orbitrap System: Ultimate 3000 UHPLC (Dionex, USA), and Q Exactive high resolution mass spectrometry (Thermo Fisher Scientific, USA); methanol and acetonitrile (HPLC) (Thermo Fisher Scientific Inc.), and formic acid (HPLC) (Aladdin Industrial Co., Ltd.).

Shuanghua Baihe tablet (Lot No. 42070405, 0.6 g/tablet, Yangzte River Pharmaceutical Co., Ltd., China) was dissolved in 0.5% carboxymethylcellulose sodium (CMC-Na) aqueous solution. Dexamethasone (Zhejiang Xianju Pharmaceutical Co., Ltd., China) was administered at a dose of 1 mg·kg⁻¹. 5-Fluorouracil ≥ 99% (HPLC) (Sigma-Aldrich) was administered at a dose of 40 mg kg⁻¹ at a concentration of 50 mg mL⁻¹. Ultra-pure water was prepared with the Milli-Q water purification system (Millipore, MA, USA).

Male Sprague-Dawley (SD) rats (6–8 weeks, 180–220 g) were purchased from the Experimental Animal Center of Henan Province (Zhengzhou, China). All animals were fed under constantly standard environmental conditions (23 ± 1 °C, 55% ± 5% humidity and a 12/12 h light/dark cycle) for one week before experiments. Ethical approval for the animal experiments were obtained from the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University (No. 2020-ky-336)

Establishment of an OM model

In order to simulate the immunosuppressive effect induced by chemotherapy, rats were given 40 mg·kg⁻¹ 5-FU solution on the -3 and -1 days of the experiment, while those in the normal control (NC) group were intraperitoneally injected (i.p.) with an equal proportion of normal saline. On day 0, the rats were anesthetized with 10% chloral hydrate (3 mL·kg⁻¹, i.p.). Then, 0.1 mL of 10% acetic acid was injected into the left side of the rat’s mouth buccal mucosa, while those in the NC group were injected with the same amount of normal saline. The rats injected with 10% acetic acid formed oral ulcers after 24 h [13, 14]. After ten days of administration, all the animals were euthanized with thiopental (80 mg·kg⁻¹, i.p.) [15] (Fig. 2A).

Experimental design and sample collection

A total of 30 SD rats were randomly divided into the following groups (n = 6): a normal control (NC) group, a model control (MC) group, SBT 0.75, 3 g·kg⁻¹ groups (S1, S3) (0.75 g·kg⁻¹ as the clinical equivalent dose), a positive drug (D) group (dexamethasone 1 mg·kg⁻¹). The NC group, not subjected to OM modeling, was intragastrically administered with 0.5% CMC-Na aqueous solution, once per day for 10 days. The MC group, subjected to OM modeling by 5-FU and 10% acetic acid, received 0.5% CMC-Na aqueous solution (i.g.) daily for 10 days. The S1, S3 and D groups, subjected to OM modeling, were given corresponding drugs (i.g.) once per day until the end of the experiment.

Then, about 4 mL of blood sample was collected from the abdominal artery of rats and divided into two parts. One part was coagulated at room temperature for 1 h, before centrifugation at 1 000 g 4 °C for 10 min to obtain the serum. The other part was stored in a heparinized EP tube, before centrifugation at 1 000 g 4 °C for 10 min to obtain the plasma. The plasma and serum were stored at 80 °C for later use. After blood collection, the spleen and thymus were taken and weighted to calculate their indexes. Spleen index = spleen mass/body mass (mg·g⁻¹). Thymus index = thymus mass/body mass (mg·g⁻¹). Then, the OM tissues of rats were collected and divided into two parts: one part was stored in 80 °C and the other was immersed in 10% (V/V) neutral buffered formalin solution for histological analysis.

Biochemistry tests

Macrosopic and histopathological analysis

The oral mucosa of rats were exposed, photographed, and scored according to the following scoring criteria: 0, normal mucosa, without chyme or vascular dilation; 1, erythema, without erosion; 2, severe erythema, vascular dilatation and surface erosion; 3, one or more ulcers, the total area of the ulcers was less than 25%, severe erythema and vascular dilation; 4, the total number of cheek pouch ulcers was about 50%; and 5, the mucosa of cheek pouches was almost completely ulcerated and the mucosa lost its flexibility [16].

For histological examination, the tissue samples of oral mucosa was embedded in paraffin blocks and cut into 3-μm thick sections, which were stained with hematoxylin and eosin (HE). The histopathological evaluation also utilized a scoring method, which determined inflammatory cell infiltration, vasodilation, bleeding areas, edema and abscesses in a single blind manner. The scoring criteria were listed as follows: 0, normal epithelium and connective tissue; 1, discrete areas of vasodilation or reepithelialization, and mild inflammation infiltration; 2, moderate vascular dilatation, epithelial degeneration, and inflammatory infiltration of neutral granulocytes, with the presence of bleeding areas, edema, and ulcers, but not abscess; 3, severe vasodilation and neutrophilic inflammation [17].

Flow cytometry

After the rats were euthanized, the spleens were immediately removed from the abdominal cavity, which were sufficiently ground and passed through a 40 μm filter to prepare splenic cell suspension. Red blood cells (RBCs) in the splenic cell suspension were fully lysed with RBC lysis solution. The resultant cell suspension was incubated with FITC anti-rat CD4 and APC anti-rat CD25 (Biologend Biotechnology Co., Ltd., USA), while PE anti-rat Foxp3 antibody was used for intracellular staining according to the manufacturer’s instructions (Biologend Biotechnology Co., Ltd., USA). Then, the samples were collected by flow cytometry (ACEA Novocyte3130, USA) according to the instructions, and data analysis were conducted with FlowJO software version 10.0 (Tree Star, Inc. Ashland, OR, USA).

Cytokine quantification

Rat TNF-α enzyme-linked immunosorbent assay kit (Multisciences Biotech, Co., Ltd., China) and rat IL-17 ELISA kit (Thermo Fisher Scientific Co., Ltd., USA) were
used to perform cytokine quantification, and the absorbance was detected at 450 nm. The frozen serum was melted on ice. Then, the content of TNF-α in the plasma of different groups was detected according to the manufacturer’s instructions.

**RNA extraction and qRT-PCR analysis**

Total RNA was extracted from the tissues using Trizol (Invitrogen, Carlsbad, CA, USA) in accordance with instruction manual. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by transfer into RNA-free EP tubes and storage at −80 °C. Complementary DNA (cDNA) was synthesized from total RNA using the PrimeScript RT Reagent Kit (Takara, Dalian, China), and PCR was performed using the SYBR® Green Premix Pro Taq HS qPCR Kit (AG11701, Accurate Biotechnology, Changsha, China). PCR was conducted on the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the \(2^{-\Delta\Delta CT}\) method. The primers used in this study are shown as following: ALOX15: F′-TTTGACAAGGCAATGAGCAC, R′-GCAGGGCGTTACTTTAGCATAG; GATM: F′-CACAGAGCAGCAGCAGAAC, R′-TCAGCTGCACAGGAATTTTG; ALAS2: F′-CCACTCAAGGACCAACCTGT, R′-GATTGGCTTCCGGGTAGTTGA; CYP1A1: F′-CAAAGCCCATGTTCCTGTTT, R′-AGCGGTTCATGACTGTACCC; PLA2G5: F′-TGAGGTTTTGTGCTTGTGAC; R′-CAGAGGAAGGCAGATCCAAG; CYP2J2: F′-ACTTAGCAGAGGGCCTGTCAG, R′-AACAAGTTGCCCACAAAGG.

**Transcriptome analysis**

**Total RNA extraction**

Total RNA was extracted from the tissues using Trizol (Invitrogen, Carlsbad, CA, USA) in accordance with instruction manual. About 60 mg of tissues were ground into...
powder after being frozen in liquid nitrogen in a 2 mL tube, which were then homogenized for 2 min and horizontally placed for 5 min. The mix was centrifuged at 12,000 g at 4 °C for 5 min, and the resultant supernatant was transferred into a new EP tube containing pre-filled 0.3 mL chloroform/isoamyl alcohol (24:1). The mix was vigorously shaken for 15 s, before centrifugation at 12,000 g at 4 °C for 10 min. Then, the upper aqueous phase where RNA remained was transferred into a new tube with equal volume of isopropyl alcohol, and then centrifuged at 13,600 r·min⁻¹ at 4 °C for 20 min. After the supernatant was removed, the RNA pellet was rinsed twice with 1 mL 75% ethanol, and then the mix was centrifuged at 13,600 r·min⁻¹ at 4 °C for 3 min. The residual ethanol was collected, and the pellet was allowed to air dry in the biosafety cabinet for 5 min. Finally, 25–100 μL of DEPC-treated water was added to dissolve the RNA. Subsequently, total RNA was quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA).

mRNA library construction

Oligo (dT)-attached magnetic beads were utilized to purify mRNA. The purified mRNA was fragmented into small pieces with fragment buffer at appropriate temperatures. Then, the first-strand cDNA was generated in First Strand reaction system by PCR, and the second-strand cDNA was generated in the same way. After the reaction product was purified by magnetic beads, A-Tailing Mix and RNA Index Adapters were added and incubated until the end was repaired. The cDNA fragments with adapters were amplified by PCR, and the products were purified by Ampure XP Beads. The qualified library was amplified on cBot to generate the cluster on the flowcell, and the amplified flowcell was sequenced single end on the HiSeq4000 or HiSeq X-ten platform (BGI-Shenzhen, China).

Data processing and analysis

The sequencing data was filtered with SOAPnuke (v1.5.2) [18] by (1) removing reads containing sequencing adapter; (2) removing reads of which the low-quality base ratio (base quality less than or equal to 5) was over 20%; (3) removing reads whose unknown base (‘N’ base) ratio was above 5%; afterwards clean reads were obtained and stored in FASTQ format. The clean reads were mapped to the reference genome using HISAT2 (v2.0.4) [19]. Apply Bowtie2 (v2.2.5) (Langmead and Salzberg, 2012) was utilized to align the clean reads with the reference coding gene set, and the mapping efficiency was selected from S1 and S3 groups as the SBT group and SBT group (six samples with the best therapeutic effect were selected from S1 and S3 groups as the SBT group) (n = 6) were taken out of refrigerator (−80 °C) and melted on ice. Plasma (50 μL) was spiked with 150 μL acetonitrile solution and 10 μL internal standard (200 ng mL⁻¹ Erlotinib-d6 and CA-d4, dissolved in methanol). After vortexing for 2 min, the mixture was centrifuged at 14,000 rpm at 4 °C for 10 min. The 150 μL supernatant was extracted and volatilized for 2 h. Then, it was reconstituted with 100 μL acetonitrile water (1:1), and centrifuged at 14,000 rpm at 4 °C for 10 min. Then, 90 μL supernatant was extracted and transferred to an automatic sampling bottle for analysis. Pooled plasma was a mix composed of 10 μL plasma of each sample, which was regarded as the quality control (QC) samples and processed in the same way.

Instrument operating conditions

The UHPLC system was utilized to separate metabolites in the plasma, and 5 μL aliquot of each sample was injected onto a ACQUITY UHPLC® BEH C₁₈ (50 mm × 2.1 mm, 1.7 μm) chromatographic column (Waters, USA) at 40 °C. Phase A was acetonitrile and phase B was 0.1% formic acid aqueous solution. Gradient elution was performed as follows at a flow rate of 0.3 mL·min⁻¹: 0–1.0 min, 95% A; 1.0–9.0 min, 95%–0% A; 9.0–12.0 min, 0%–0% A; 12.0–12.1 min, 0%–95% A; and 12.1–15.0 min, 95% A.

The spray voltage of electrospray ionization (ESI) source was set to +3.5 kV and -2.8 kV in positive and negative ion modes respectively. The data were scanned in Full/ddmms patterns with a range of 80.00–1200.00 m/z, a first-order MS resolution of 70 000, a second-order MS Resolution of 17 500. The gradient collision energy was set to 20, 30 and 40 eV. The capillary temperature and the auxiliary gas flow were 320 °C and 30 arb, respectively. For the sake of the reliability of the results, the random sampling method was used to inject samples, and the instrument was balanced with 5 QC samples before testing. Then, one QC sample was inserted between every five samples to evaluate the stability of the apparatus.

Data processing and analysis

The original data from plasma samples were imported into the Compound Discoverer (Version 3.0, Thermo Scientific) for peak detection, calibration, and normalization pretreatment. After the data was preprocessed, principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed on the two-dimensional data matrix with the SIMCA software (version 14.1, Umetrics AB, Umea, Sweden). Then, the risk of model overfitting was evaluated by a 200-time permutation test. In order to further screen the potential markers between different groups, the Student’s t-test was performed on all detected metabolite peaks to calculate their variable importance in the projection (VIP) and fold change values. VIP > 1 and P < 0.05 metabolites were used as potential markers, which were identified by searching the HMDB, MoNA and KEGG databases and comparing their m/z value, retention time and MS/MS spectra using MassFrontier. A heat map was drawn...
to show the variation trend of the identified key metabolites with the heatmap package (R version 3.3.0). Finally, the KEGG and MetaboAnalyst database were chosen to uncover the metabolic pathways associated with SBT.

**Statistical analysis**

SPSS16.0 software was utilized to conduct statistical analysis. The student’s t-test was used for comparison between groups. Measurement data were expressed as mean ± standard deviation, and $P < 0.05$ indicated statistical significance.

**Results**

**Macroscopic and histopathological analysis**

Before intragastric administration, there was no obvious difference in the ulcer area and erosion degree of the rats’ oral mucosa among the groups. After the experiment, the general morphology of the oral ulcer surface was observed, and the ulcer mucosa tissue was taken for HE staining. The oral mucosa of rats in the NC group was normal, without inflammatory cell infiltration. The rats of the MC group showed about 50% oral mucosal ulcers, oral mucosal damage, squamous epithelium breakage and shedding, and the surface covered with necrotic tissue (Figs. 2B, 2C). According to HE staining, there was serious inflammatory infiltration of neutrophils, pyknosis, rupture, and deep staining of the nuclei. Rats in the S1 group presented no signs of mucosal erosion, superficial erosion, mild abscess, and discrete inflammatory cell infiltration. Compared with the MC and S1 groups, the ulcer area in the S3 and D groups significantly decreased, with mild superficial erosion, discrete inflammatory cell infiltration, but without bleeding, edema or abscess (P < 0.05, Figs. 2D, 2E).

**Effects of SBT on the thymus and spleen indexes of OM rats**

The spleen is the largest immune organ of the human body and plays a vital role in regulating immune function of the body. Therefore, we further calculated the spleen index and thymus index of rats. Spleen index = spleen mass/body mass (mg·g$^{-1}$); thymus index = thymus mass/body mass (mg·g$^{-1}$). Compared with the NC group, the thymus index and spleen index of rats in the MC group significantly increased (P < 0.01). Compared with the MC group, the thymus index and spleen index of rats in the S1 and S3 groups reduced to various degrees. Compared with the NC group, the thymus index and spleen index of rats in the S3 group reduced, with statistical difference (P < 0.05, Table 1). These findings indicated that SBT may relieve splenic and thymus hypertrophy, and reduce inflammatory stress response.

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>MC</th>
<th>S1</th>
<th>S3</th>
</tr>
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<tbody>
<tr>
<td>Spleen index</td>
<td>1.97 ± 0.12</td>
<td>4.89 ± 0.11</td>
<td>3.53 ± 0.44</td>
<td>2.55 ± 0.40</td>
</tr>
<tr>
<td>Thymus index</td>
<td>0.95 ± 0.06</td>
<td>1.97 ± 0.14</td>
<td>1.58 ± 0.11</td>
<td>1.23 ± 0.16</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 vs the NC group; ***P < 0.05, ****P < 0.01 vs the MC group.

Transcriptomic analysis reveals alterations in gene expression in OM that are restored by SBT treatment

**Transcriptomics alteration in the NC, MC and SBT groups**

In light of the better treatment effects of the SBT high-dose group, the tissue samples were taken for transcriptomics analysis to investigate the gene expression and pathway changes caused by SBT. First, we compared the gene expression of different samples, which indicated that the distribution of gene expression in different samples was generally the same (Fig. 3A). To identify transcriptome changes in the NC, MC and SBT groups, principal component analysis (PCA) was conducted to examine the gene expression relationships among the NC, MC and SBT groups. Each replicate from the same group was closely clustered together, which implied that the repeatability of each treatment was consistent with our expectation. The MC and SBT groups were clustered far from the NC group, which indicated that MC and SBT groups had obvious changes in gene expression (Fig. 3B). Then, differential expression analysis between the MC and NC group identified 590 significantly dysfunctional genes ($P < 0.05$ and |log2(fold change)| ≥ 1; Fig. 3C), where the expression of 393 genes were restored in the SBT group compared with the MC group ($P < 0.05$ and |log2(fold change)| ≥ 1). The 393 genes were defined as reversed genes (Table S1). The heat map showed the reversed genes with significant differences (Fig. 3D, P < 0.01).

**Enrichment analysis of reversed genes**

To understand the pathophysiological relevance of the reversed genes and the impact of SBT on their expression, these genes were subjected to GO and KEGG pathway analyses. Interestingly, biological processes (BP) enrichment analysis carried out by ClueGO showed that the reversed genes mainly participated in processes related to inflammatory and immune response, which play a key role in the occurrence and development of OM (Fig. 3F). KEGG pathway enrichment analysis was performed to illustrate the functions of these reversed genes (Fig. 4A). These reversed genes were highly enriched in the following functions and pathways: IL-17 signaling pathway, leukocyte transendothelial migration, glycosphingolipid biosynthesis lacto and neolacto series, cytokine-cytokine receptor interaction, tight junction, chemokine signaling pathway, TNF signaling pathway, intestinal immune network for IgA production, T cell receptor signaling pathway and cAMP signaling pathway. GO and KEGG enrichment analyses tend to focus on genes with significant differences and omit some genes with insignificant differential expression but biological significance, GSVA was performed to screen out important differential pathways. The results indicated that ether lipid metabolism, glycine serine and threonine metabolism, alpha linolenic acid metabolism and valine leucine and isoleucine degradation were restored by SBT treatment (Figs 4B–4E). These results confirmed that inflammatory, immune and metabolites pathway may play crucial parts in the treatment of OM with SBT.
Target metabolites analysis

We further explored the expression of metabolites for the NC, MC and SBT groups using a metabolome method. The data processed by the Compound Discovery software was submitted to the SIMCA software and all samples were subjected to principal component analysis (PCA). It was observed that the QC samples stayed together in the positive ion and negative ion mode, indicating that the metabolic spectrum analysis method had good stability and repeatability (Figs. 5A, 5B). Moreover, PCA results showed that there was certain differences among the MC, NC and SBT groups. In order to establish the metabolic markers for discrimination between NC and MC groups, an OPLS-DA model was established, which demonstrated that the separation between the NC and MC groups presented in the scatter plot of the model was quite evident (Figs. S1A, S1D). After a 200 times’ permutation, the resultant $R^2Y$ and $Q^2$ values of the obtained model ($R^2Y = 1$, $Q^2 = 0.625$, Fig. S1B; $R^2Y = 1$, $Q^2 = 0.552$, Fig. S3E), showed that the model was stable and reliable, without overfitting. Furthermore, the value above 1.0 was considered to be significant in the VIP plot (Figs. S1C, S1F). Then, in order to ensure the statistical significance and the concentration change status, the student’s $t$-test and fold change values were also calculated. Based on the filter of VIP > 1.0 and $P < 0.05$, a total of 32 metabolites were identified, including cholic acid, linoleic acid, 4-pyridoxic acid, and LysoPC by referencing to previous reports [22-25] and comparing with several databases (Table 2). A heat map was drawn based on 32 differential metabolites presented in the
NC and MC groups, indicating that there was an obvious demarcation line between the NC group and MC group (Fig. 5C). Among the identified substances, 18 of them were reversibly regulated by SBT in the SBT group and even achieved the level in the NC group (Table 2), which were also shown in a heat map (Fig. 5D).

**Pathway analysis**

Based on the differential metabolites between the NC group and MC group, a biology analysis on the metabolic pathways was conducted. The results demonstrated that the majority of metabolic disorders induced by OM mainly included linoleic acid metabolism, porphyrin and chlorophyll metabolism, biosynthesis of unsaturated fatty acids, glycine, serine and threonine metabolism, arginine and proline metabolism, valine, leucine and isoleucine biosynthesis, and vitamin B6 metabolism etc. (Fig. 5E). After the treatment of SBT, 18 disordered metabolites were adjusted in reverse, which mainly participated in linoleic acid metabolism, glycerophospholipid metabolism, valine, leucine and isoleucine biosynthesis and glycine, serine and threonine metabolism (Fig. 5F). These results were consistent with the results of transcriptional sequencing enrichment analysis, which further indicated that SBT may act an excellent therapeutic role for OM patients through regulating the metabolic pathways.

**Inflammation-related indicators**

In accordance with the results of KEGG enrichment analysis, we focused on the IL-17 signaling pathway and the TNF signaling pathway. In this study, the IL-17 signaling pathway and the TNF signaling pathway were identified as the interest target for further investigation, based on the following reasons: (1) IL-17 is an important pro-inflammatory cytokine secreted by helper T cells (Th17) and innate immune cells, and plays a key role in a variety of inflammatory responses and the pathological process of autoimmune diseases [26]; and (2) TNF-α, mainly secreted by macrophages, is a cytokine involved in systemic inflammation and one of many cytokines responsible for acute response [27]. We tested the IL-17 and TNF-α content in rat serum. Compared with the NC group, the serum IL-17 and TNF-α levels in the MC group evidently increased (P < 0.01), indicating that the MC group had significant inflammatory symptoms. Compared with the MC group, the IL-17 and TNF-α levels in the serum of rats in the S1 and S3 groups evidently reduced (P < 0.05, Figs. 6A, 6B). These findings suggested that SBT can reduce the level of serum TNF-α in OM rats, thereby relieving inflammatory response. This may be one of the mechanisms of SBT to treat oral ulcer. Given that KEGG analysis results indicated cytokine-cytokine receptor interaction and T cell receptor signaling pathway, we further tested the proportion of Treg cells with immunosuppressive function in the spleen. Compared with the MC group, the proportion of Treg cells in the spleen of rats in the S1 and S3 groups significantly increased (P < 0.01, Fig. 6C), which further suggested that SBT can maintain immune balance of rats and play an essential role in the recovery of OM rats.

**Biological model integrating transcriptome and metabolome data**

Our metabolome data showed that SBT treatment reversed the disorder of linoleic acid metabolism, glycerophospholipid metabolism, valine, leucine and isoleucine biosynthesis and glycine, serine and threonine metabolism. Moreover, the enrichment results of transcriptome sequencing data indicated that glycosphingolipid biosynthesis, ether lipid metabolism, glycine serine and threonine metabolism,
alpha linolenic acid metabolism and valine leucine and isoleucine degradation also participated in SBT treatment, which was consistent with metabolomics alterations. Then, with an integrative correlation network of the transcriptome (reversed genes, \( P < 0.05 \)) and metabolome (reversed metabolites, \( P < 0.05 \)), we explored the underlying relationship between targets and metabolites during the treatment of OM by SBT. This network revealed three metabolism pathways, including linoleic acid metabolism, glycerophospholipid metabolism and amino acid metabolism, and 13 targets were involved in these metabolism pathways (Fig. 7A). The targets involved in linoleic acid metabolism including ALOX15, FADS1, CYP2J2 and CYP1A1, and glycerophospholipid metabolism including PISD, ALOX15 and SAT1. Targets related to amino acid metabolism including CNDP2, SAT1, GATM, ALAS2, PC, EHHADH, BCAT2 and PLA2G5. In addition, ALOX15, GATM, ALAS2, CYP1A1 PLA2G5 and CYP2J2 were related with immunoreaction and inflammatory reaction, which was consistent with the results of enrichment analysis. In order to confirm RNA-seq results, qRT-PCR analysis of the six genes was further conducted using OM tissue, and the results were consistent with those from RNA-seq analysis (Fig. 7B). The comprehensive results indicated that both the genes and metabolites of linoleic acid metabolism, glycerophospholipid metabolism and amino acid metabolism play an important role in the treatment of OM with SBT, and the metabolism disorders are correlated with the occurrence and development of the disease.

**Discussion**

At present, the mechanism of OM is not completely understood. The incidence of OM is increasing globally, and more and more patients with malignant tumors suffer from OM after radiotherapy or chemotherapy \(^1\,^{1,\,28}\). Therefore, im-
Table 2: Different metabolites identified between the NC vs MC groups and reversed in the SBT group

<table>
<thead>
<tr>
<th>No.</th>
<th>HMDB ID</th>
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<th>Formula</th>
<th>Molecular Weight</th>
<th>t/min</th>
<th>Name</th>
<th>VIP</th>
<th>NC vs MC</th>
<th>SBT vs MC</th>
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<td>C01595</td>
<td>C18H32O2</td>
<td>280.239 93</td>
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<td>C02710</td>
<td>C8H15NO3</td>
<td>173.104 41</td>
<td>4.685</td>
<td>N-Acetylleucine</td>
<td>1.570 28</td>
<td>*(↑)^</td>
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<td>3</td>
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<td>C16533</td>
<td>C2H2H4O2</td>
<td>336.303 06</td>
<td>11.423</td>
<td>Docosadienoate</td>
<td>1.846 82</td>
<td>*↑(↑)</td>
<td>−3.48</td>
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<td>HMDB0046677</td>
<td>C14762</td>
<td>C18H32O3</td>
<td>296.235 18</td>
<td>9.216</td>
<td>13-HODE</td>
<td>1.650 88</td>
<td>*(↑)^</td>
<td>−1.27</td>
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<td>5</td>
<td>HMDB0000355</td>
<td>C03761</td>
<td>C6H10O5</td>
<td>162.052 92</td>
<td>1.065</td>
<td>3-Hydroxyphenylglycine</td>
<td>1.789 04</td>
<td>**(↑)^</td>
<td>−0.268</td>
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<td>HMDB0010386</td>
<td>C04230</td>
<td>C2H6H5NO7P</td>
<td>519.333 39</td>
<td>9.08</td>
<td>LysoPC (18 : 2 (9Z,12Z,15Z) : 0)</td>
<td>1.918 77</td>
<td>**(↑)^</td>
<td>−1.145</td>
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<td>HMDB0000637</td>
<td>C05466</td>
<td>C2H6H4NO5</td>
<td>449.313 97</td>
<td>5.969</td>
<td>Chenodeoxycholic acid conjugate</td>
<td>1.840 56</td>
<td>*↑(↑)</td>
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<td>C00847</td>
<td>C8H9NO4</td>
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<td>1.079</td>
<td>4-Pyridoxic acid</td>
<td>1.791 13</td>
<td>*(↑)^</td>
<td>0.466</td>
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<td>HMDB0003464</td>
<td>C01035</td>
<td>C5H11NO2</td>
<td>145.085 3</td>
<td>0.999</td>
<td>4-Guanidinobutanoic acid</td>
<td>1.731 35</td>
<td>*(↑)^</td>
<td>0.252</td>
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<td>HMDB000123</td>
<td>C01018</td>
<td>C7H7NO2</td>
<td>137.047 85</td>
<td>0.991</td>
<td>2-Aminobenzoic acid</td>
<td>1.755 02</td>
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<td>0.398</td>
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<td>11</td>
<td>HMDB0055060</td>
<td>C16525</td>
<td>C20H36O2</td>
<td>308.271 49</td>
<td>10.767</td>
<td>Eicosadienoic acid</td>
<td>1.713 93</td>
<td>*(↑)^</td>
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<td>HMDB0000827</td>
<td>C01530</td>
<td>C18H36O2</td>
<td>284.271 34</td>
<td>11.372</td>
<td>Stearic acid</td>
<td>1.808 05</td>
<td>*(↑)^</td>
<td>0.221</td>
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<td>13</td>
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<td>C04230</td>
<td>C2H5H4NO7P</td>
<td>547.324 11</td>
<td>9.075</td>
<td>LysoPC (20 : 2 (11Z,14Z) : 0)</td>
<td>1.690 18</td>
<td>*(↑)^</td>
<td>−0.394</td>
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<td>C00300</td>
<td>C4H9NO3</td>
<td>131.069 6</td>
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<td>1.574 37</td>
<td>*(↑)^</td>
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<td>C04230</td>
<td>C2H4H8NO7P</td>
<td>493.316 92</td>
<td>8.025</td>
<td>LysoPC (16 : 1 (9Z) : 0)</td>
<td>1.851</td>
<td>*(↑)^</td>
<td>−1.947</td>
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<td>HMDB0002343</td>
<td>C14772</td>
<td>C20H34O4</td>
<td>355.272 12</td>
<td>6.905</td>
<td>5, 6-DHET</td>
<td>1.716 83</td>
<td>*(↑)^</td>
<td>−1.335</td>
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<td>HMDB0000904</td>
<td>C00158</td>
<td>C6H10O7</td>
<td>192.026 19</td>
<td>1.229</td>
<td>Citric acid</td>
<td>1.761 73</td>
<td>*(↑)^</td>
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<td>C04230</td>
<td>C2H6H5NO2P</td>
<td>521.348 1</td>
<td>8.872</td>
<td>LysoPC (18 : 1(11Z) : 0)</td>
<td>1.792 77</td>
<td>*(↑)^</td>
<td>−0.49</td>
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<td>341.162 8</td>
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<td>Naltrexone</td>
<td>1.683 17</td>
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<td>C00486</td>
<td>C33H36N4O6</td>
<td>584.264 06</td>
<td>11.004</td>
<td>Bilirubin</td>
<td>1.759 45</td>
<td>*(↑)^</td>
<td>−3.426</td>
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<td>21</td>
<td>HMDB0009213</td>
<td>C00350</td>
<td>C3H7H7NO7P</td>
<td>695.487 2</td>
<td>1.001</td>
<td>PE (18 : 4 (6Z,9Z,12Z,15Z) : 16 : 0)</td>
<td>1.873 56</td>
<td>*(↑)^</td>
<td>4.527</td>
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<td>22</td>
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<td>C02724</td>
<td>C18H26O2</td>
<td>274.193 13</td>
<td>7.721</td>
<td>Nandrolone</td>
<td>1.911 12</td>
<td>*(↑)^</td>
<td>4.259</td>
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<tr>
<td>23</td>
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<td>C16533</td>
<td>C2H2H4O2</td>
<td>336.303 06</td>
<td>11.423</td>
<td>Docosadienoate (22 : 2n6)</td>
<td>1.881 09</td>
<td>*(↑)^</td>
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<td>24</td>
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<td>C08493</td>
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<td>145.052 91</td>
<td>4.482</td>
<td>Indole-3-carboxaldehyde</td>
<td>1.999 42</td>
<td>*(↑)^</td>
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<td>C04230</td>
<td>C30H52NO7P</td>
<td>569.348 44</td>
<td>8.664</td>
<td>LysoPC (22 : 5 (7Z,10Z,13Z,16Z,19Z) : 0)</td>
<td>1.701 7</td>
<td>*(↑)^</td>
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<td>26</td>
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<td>C16526</td>
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<td>310.287 13</td>
<td>11.361</td>
<td>11Z-Eicosanoic acid</td>
<td>2.052 84</td>
<td>*(↑)^</td>
<td>3.567</td>
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<td>C00430</td>
<td>C5H9NO3</td>
<td>131.058 39</td>
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<td>5-Aminolevulinic acid</td>
<td>1.825 88</td>
<td>*(↑)^</td>
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<tr>
<td>28</td>
<td>HMDB0010394</td>
<td>C04230</td>
<td>C28H52NO7P</td>
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<td>8.591</td>
<td>LysoPC (20 : 3 (8Z,11Z,14Z) : 0)</td>
<td>1.824 76</td>
<td>*(↑)^</td>
<td>3.063</td>
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<tr>
<td>29</td>
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<td>C04483</td>
<td>C24H4O4</td>
<td>392.292 61</td>
<td>7.718</td>
<td>Deoxycholic acid</td>
<td>1.786 83</td>
<td>*(↑)^</td>
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<td>C00695</td>
<td>C24H4O5</td>
<td>408.287 58</td>
<td>6.212</td>
<td>Cholic acid</td>
<td>1.850 65</td>
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<td>HMDB0000687</td>
<td>C00123</td>
<td>C6H13NO2</td>
<td>131.094 68</td>
<td>1.095</td>
<td>L-Leucine</td>
<td>1.565 64</td>
<td>*(↑)^</td>
<td>0.037</td>
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**Fig. 6** The biochemistry analysis in the NC, MC and SBT groups. The levels of IL-17(A) and TNF-α (B) in different groups; (C) the proportion of Treg cells in the spleen of different groups (*P < 0.05, **P < 0.01)

**Fig. 7** Biological model integrating transcriptome and metabolome data. (A) Metabolite pathways, metabolites and genes perturbations in the NC, MC and SBT groups. (The histogram indicated the content of metabolites in different groups. The point shown the Log2FC value for the difference analysis of different groups); (B) Expression of the reversed genes related to metabolism, immunoreaction and inflammatory reaction as determined by qRT-PCR analysis of OM tissues (*P < 0.05; **P < 0.01)
proving the prevention and treatment of OM caused by radiotherapy and chemotherapy is of clinical importance to prolong the survival time of patients, improve the quality of life, reduce the economic burden of patients and improve the quality of cancer care. As a TCM formula, Shuanghua Baihe tablet can regulate the body’s immunity, and exert anti-inflammatory and analgesic effects, which can effectively alleviate the symptoms of mucosal inflammation and oral odor. In recent years, a series of studies have proved that SBT exhibits good effects in the treatment of chemotherapy-induced OM. It can not only relieve the pain of patients, but also significantly promote the healing of congestive erosions. However, due to the complex active ingredients of TCM compounds and the unclear target of action, the mechanism of SBT in the treatment of OM has not been elucidated.

With the development of multi-omics technology, it is possible to explore the mechanism of Chinese herbal compound prescriptions. In this study, we investigated the synergistic effects of the main active components of SBT and explored its potential mechanism. The comprehensive treatment effect of SBT was observed according to ulcer score, as well as histopathology, biochemistry and other indexes, which indicated that SBT exhibited good therapeutic effects on OM caused by chemotherapy. In order to investigate the underlying mechanism of SBT in the treatment of OM, we compared the transcriptional profiles of the NC and MC groups’ tissues with the identified dysregulated genes of OM. After the treatment of SBT, several of dysregulated genes were reversed. Gene ontology and pathway enrichment analysis indicated that these reversed genes had notable correlation with immune response, cytokine–cytokine receptor interaction, leukocyte transendothelial migration, IL-17 signaling pathway, TNF signaling pathway and biological metabolic process. With the treatment of SBT on OM, the immune and inflammation process of cells was also severely affected, thus leading to IL-17, TNF-α and Treg cells disturbances. The results were verified by flow cytometry and cytokine quantification. Previous studies showed that IL-17, TNF-α and Treg cells were closely related to the progression of OM, whose proportion determined the recovery rate and recurrent rate of OM. Besides, some disordered metabolic processes contributed to preterm delivery. The comprehensive treatment effect of SBT was observed by metabolomic analysis which can explore metabolic profile of metabolic pathway, verifying transcriptional variability, and thus help biologists comprehend the mechanism of the treatment of OM by SBT.

Conclusion

In conclusion, this study is the first to investigate the therapeutic effect of SBT on an OM model in rats by combining transcriptomics and metabolomics. The results show that SBT can improve the inflammatory symptoms of oral mucosa, which may be related to its anti-inflammatory function, maintaining immune balance of the body and repairing the metabolic pathways of many disorders. Notably, immune-related pathways, such as IL-17 signaling pathway, TNF signaling pathway and metabolic-related pathways like linoleic acid metabolism play essential roles in the SBT treatment on OM.

Availability of data and materials

Processed data is contained within the article. Raw data is available from the corresponding author upon request.

Abbreation

OM, Oral mucositis; SBT, Shuanghua Baihe tablet; TCM, traditional Chinese medicine; IL-17, interleukin-17; TNF-α, tumor necrosis factor-α; CMC-Na, Carboxymethylcellulose sodium; NC, normal control; MC, model control; HE, hematoxylin and eosin; RT-qPCR, real-time quantitative PCR; GO, Gene Ontology; GSVA, Gene Set Variation Analysis; ESI, Electrospray ionization; VIP, variable importance in the projection; PCA, Principal component analysis
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