

Transcriptome Sequencing Explores the Mechanism of Baicalin on Bone Cancer Pain

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Introduction: Bone cancer pain is characterized by persistent pain, usually requiring drugs to relieve pain. Baicalin, a flavonoid compound extracted from *Scutellaria baicalensis*, which has antioxidant and analgesic effects. But, the effect of baicalin on bone cancer pain is unclear. Thus, this study aimed to explore the mechanism of baicalin on SD rats with bone cancer pain.

Materials and Methods: The MADB-106 breast cancer cells-induced bone pain model was constructed and carried out baicalin treatment. The therapeutic effect of baicalin on bone cancer pain model was observed by hematoxylin-eosin staining and immunofluorescence staining. We also performed transcriptome sequencing analysis of baicalin in the treatment of bone metastases. Also, RT-qPCR and ELISA were used to detect the expression levels of inflammation factors.

Results: After baicalin treatment, osteoclast activation was inhibited and the number of bone trabeculae was increased. Baicalin inhibited the protein expression level of inflammatory factors (IL-1 β , IL-6, TNF- α and PGE2) in the bone metastases group. Based on the transcriptome sequencing of the bone metastases group and the baicalin treatment group, baicalin inhibited the expression of *ALPP*, *DUSP1*, *CYR61*, *ALPPL2*, *SPP1* and *TLR4*. RT-qPCR was also used to validate the expression levels of these cytokine genes.

Conclusion: Baicalin had a certain inhibitory effect on the SD rat model of bone metastasis cancer. These insights can guide future research on the molecular mechanism of bone cancer pain and provide a theoretical basis for baicalin in the treatment of bone pain caused by breast cancer in the future.

Keywords: breast cancer, bone cancer pain, baicalin, transcriptome sequencing

Introduction

With the continuous improvement of cancer treatment technology, the 3-year and 5-year survival rates of cancer patients have been greatly improved. However, cancer still cause pain to the patients, which seriously affects their quality of life. This is because in many common cancers, tumors can easily metastase to the spine, hips, ribs, femur and tibia,¹ and then invade the surrounding soft tissue. Usually, tumor metastasis to bone will activate osteoclasts and lead to bone resorption, resulting in moderate to severe persistent pain.^{2,3} According to statistics, about 60–90% of patients with advanced cancer have suffered from varying degrees of pain, of which about 30% have suffered from continuous severe pain,⁴ which may be related to bone metastasis. As report went, up to 70% of breast and prostate cancer patients and up to 30% of thyroid, bladder and lung cancer patients had bone metastases.⁵

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Currently, the treatment of pain in bone metastasis involved the use of a variety of adjuvant methods, including surgery, radiotherapy, chemotherapy, as well as drug treatment-bisphosphonates, calcitonin, analgesics. Nevertheless, the side effects of some drugs limit their clinical application, such as gastric ulcer and nephrotoxicity of non-steroidal anti-inflammatory analgesics.⁶ In recent years, Chinese herbal extracts have attracted the attention of many researchers. Baicalin (7-d-glucuronic acid, 5,6-dihydroxyflavone) is a flavonoid extracted from *Scutellaria baicalensis* and other Chinese herbal medicines. Baicalin has anti-tumor,⁷ anti-inflammatory,⁸ neuroprotective⁹ and anti-anxiety effects.¹⁰ In previous studies, baicalin can significantly inhibit the proliferation of human prostate cancer,¹¹ and induce the apoptosis of human colorectal cancer cells SW620.¹² Also, Wang et al revealed that baicalin suppressed the invasion and metastasis of human osteosarcoma cells by EMT induced by TGF- β 1.¹³ However, the treatment mechanism of baicalin on bone metastases cancer has not been studied yet.

In the study, we explored the therapeutic effect of baicalin on bone metastasis by constructing SD rat model of bone metastasis cancer and carrying out baicalin treatment. And we used transcriptomics sequencing technology and RT-qPCR verification experiments to explore the treatment mechanism of baicalin on bone metastasis cancer. We hope to provide a theoretical basis for baicalin in the treatment of bone metastases=cancer.

Methods

Cell Culture

MADB-106 breast cancer cells were purchased from the Beijing Union Medical College Cell Center, and the cells were cultured into DMEM/F12 medium containing 10% FBS in a 37°C 5% CO₂ incubator. When the cells reached the logarithmic phase (70–80%), trypsin was used to digest cells and collected cell suspension. After centrifugation, a cell suspension with a concentration of 1×10^5 cells/10 μ L final dilution was prepared and put it on ice for standby.

Induction of Bone Cancer Pain and Treatment with Baicalin

SD rats were randomly divided into the NC group and the Bone metastases group after adaptive feeding for one week. SD rats were anesthetized with pentobarbital sodium (50 mg/kg). In a simple fixation device, the legs

of SD rats were fixed with clips and the left leg was shaved. After disinfection with 70% v/v ethanol, the skin incision was parallel to the tibia. Drill a hole in the left tibial plateau with the disposable blood collection needle, and the breast cancer cell suspension (1×10^5 cells) diluted with 50 μ L normal saline was slowly injected into the bone marrow cavity by a disposable sterile injection needle. The control group was injected with the same amount of normal saline into the bone marrow cavity.^{14,15} In order to prevent cells from leaking out of the bone, the intraosseous injection hole was closed with bone wax and the tumor cells left outside the bone marrow cavity were removed with 70% v/v ethanol. The animals were placed on a warm mat for recovery and then put in separate cages. During the whole operation, the aseptic operation was strictly followed. In addition, after injection, some SD rats with bone cancer pain were intraperitoneally injected with baicalin (30 mg/kg) every 2 days for 15 days.

Real-Time Fluorescence Quantitative PCR

Firstly, total RNA was extracted from the tumor tissues in bone marrow taken at 1 month by Tissues RNA Miniprep Kit (BW-R1-02, Biomiga) and was stored at -20°C . cDNA was synthesized by the PrimeScriptTM RT reagent Kit with gDNA Eraser (AJ51485A, Takara). The amplification reaction was completed by Hieff[®] qPCR SYBR[®] Green Master Mix (Low Rox) (H1911331, Shanghai Yisheng Biotechnology Co., Ltd) with the primers listed in [Supplementary Table 1](#). The amplification procedure was performed by pre-denaturation at 50°C for 2 min, followed by 40 cycles of 95°C 10 sec and 60°C 30 sec. The final extension procedure was performed by 95°C 15 sec. Subsequently, the amplification curve was obtained at 60°C 1 min and 95°C 15 sec. Data were statistically analyzed with $2^{-\Delta\Delta\text{Ct}}$.

Enzyme-Linked Immunosorbent Assay

The blood sample of SD rats in the control group, the Bone metastases group and Bone metastases + Baicalin group were taken from the eyes of anesthetized mice. Enzyme linked immunosorbent assay (ELISA) was used to measure the protein level of tumor-derived cytokines (PGE2/IL-1 β /IL-6/TNF- α) in mouse serum samples. Mouse derived PGE2 ELISA Kit was purchased from Shanghai renjie biotechnology Co., Ltd. Also, mouse derived ELISA Kit (IL-1 β /IL-6/TNF- α) was purchased

from Shanghai Biyuntian Biotechnology Co., Ltd. The specific steps were as follows: (1) The whole blood collected from the anesthetized mouse was put into the prepared 96-well ELISA plate, wrapped in an aluminum foil bag and placed at room temperature for 20 min. (2) We set up the standard hole and the sample hole. Add 50 μ L of different concentrations of the standard, 50 μ L of sample to be tested into the standard hole and the sample hole, respectively. (3) Then add 100 μ L of antibody labeled with peroxidase (HRP) into the sample hole and standard hole, seal the orifice plate, and incubate in an incubator for 60 min. (4) Discard the liquid and add the washing solution for multiple washing. (5) Add 50 μ L of substrate A and B, and incubate at 37°C for 15 min. (6) The OD value of each hole at the wavelength of 450nm was detected by Thermo Scientific microplate reader.

Hematoxylin-Eosin Staining

The tibia of the inoculated side was taken about 22 days after inoculation, and the surrounding muscle soft tissue was stripped and put it into 15% hydrochloric acid formaldehyde decalcification solution for 3 days. After softening, the tissue was fixed in 70% ethanol, and then dehydrated, embedded in paraffin, sectioned and stained with hematoxylin-eosin. Tumor growth and bone structure damage were observed under microscope.

Immunofluorescence Staining

Firstly, the tissue sections were fixed with 4% paraformaldehyde for 15 min and permeated with 0.1% Triton-X 100 for 10 min. The section was incubated with anti-iNOS primary antibody (Santa Cruz Biotechnology, SC-651) overnight. The primary antibody was washed without hybridization by PBS, and then incubated with the fluorescent secondary antibody. Subsequently, the sections were immersed in tartrate resistant acid phosphatase (Trap, Servicebio, G1050) solution and counterstained with hematoxylin dye solution (Servicebio, G1004) to display the nuclei. After multiple washing and drying, anhydrous ethanol and xylene were dehydrated and sealed. Under the optical microscope (Nikon, ECLIPSE E100) and imaging system (Nikon, DS-U3), the tissue sections were examined and analyzed by Imaris 8 image analysis software.

Functional Analyses

Based on the results of the transcriptome sequencing, Gene ontology (GO) and Kyoto encyclopedia of genes

and genomes (KEGG) were used to observe the function of differentially expressed genes (DEGs) presented by Fisher's exact test using the clusterProfiler 2.2.1 version. Biological process (BP), cell composition (CC) and molecular function (MF) were included in GO annotation analysis. KEGG enrichment analysis mainly focused on the mRNAs-related signaling pathways.

Statistical Analysis

SPSS 20.0 software was used for data management and statistical analysis. The Student's t-tests was used for comparison among three groups. *p*-values obtained from all figures were two-way analysis of variance. All the data were repeated for 3 times. *p*-value <0.05 was considered to have significant differences.

Results

Baicalin Recovered the Bone Resorption in Bone Cancer Pain Rats

After breast cancer cell injection about 3 weeks, CT was used to examine the degree of tibial destruction in the NC group, the Bone metastases group and the Bone metastases + Baicalin group, including bone volume fraction (BV/TV), trabecular thickness (Tb.Th), the number of trabeculae (Tb.N) and the mean spacing of trabecular bone (Tb.Sp). Compared with the control group, BV/TV, Tb.Th and Tb.N were downregulated in the Bone metastases group. Although the trend of Tb.Sp was opposite, BV/TV, Tb.Th and Tb.N were significantly increased after baicalin treatment ([Supplementary Figure 1](#)). These results showed that the bone mineral density (BMD) was reduced and the bone trabeculae was sparse in the Bone metastases group, which proved the success of establishment of cancer pain model from the perspective of radiology. The above results showed that the bone resorption in the Bone metastases group was significantly reduced compared to the NC group. The bone resorption was recovered after Baicalin treatment ([Figure 1](#)).

Baicalin Inhibited Osteoclast Activation and Promoted the Increase of Bone Trabeculae in Bone Cancer Pain Rats

Besides, the number of trabeculae and osteoclasts in SD rats were also detected in the NC group, the Bone metastases group and the Bone metastases + Baicalin group detected by HE staining and Trap staining, respectively. Therefore, Trap is a typical marker of osteoclasts, and the results of Trap

Bone metastases

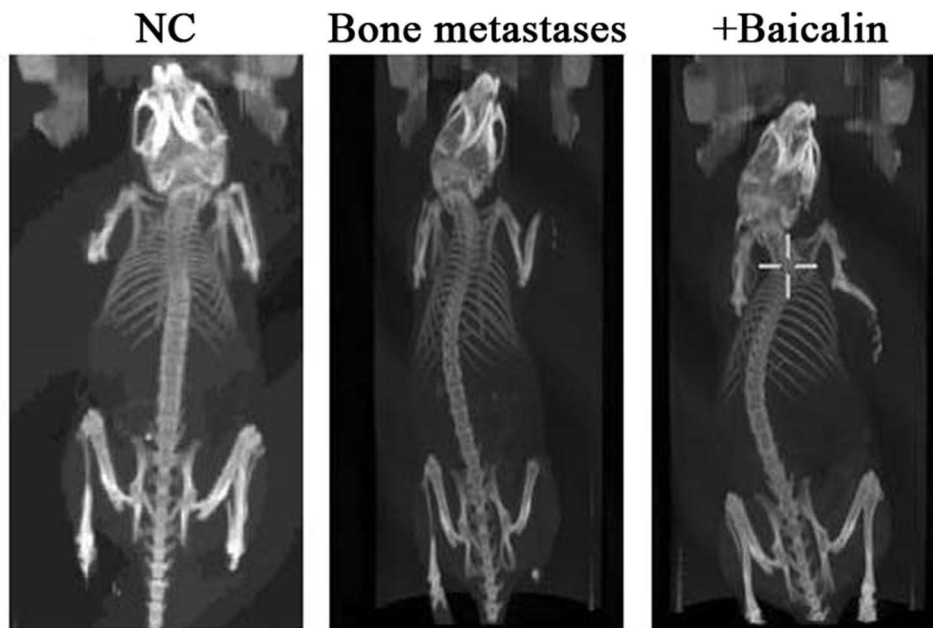


Figure 1 Baicalin recovered the bone resorption in the Bone metastases cancer. CT was used to detect BMD in SD rats of the NC group, the Bone metastases group and the Bone metastases + Baicalin group. The changes of BMD were calculated by SPSS software.

staining was that the cytoplasm of osteoclasts was wine red and the nucleus was light blue, illustrating that osteoclasts count was significantly increased in the Bone metastases group and was decreased after Baicalin treatment. HE staining also showed that the number of trabeculae decreased in the Bone metastases group and increased in the Bone metastases + Baicalin group (Figure 2).

Baicalin Inhibited the Expression of iNOS in Bone Cancer Pain Rats

NO has been reported to promote tumor metastasis, and iNOS is one of the four subtypes of NOS, which is the only rate limiting enzyme for NO synthesis. Thus, immunofluorescence detection was utilized to detect the expression of iNOS in the groups. The results revealed that the expression

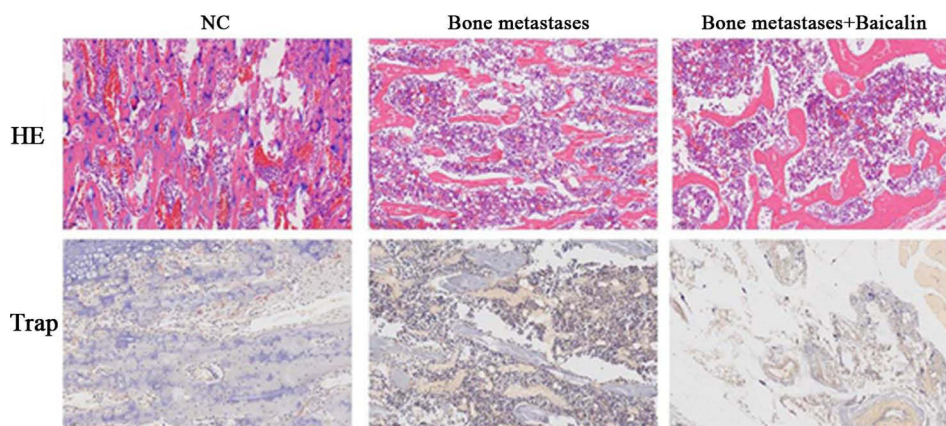


Figure 2 Baicalin inhibited osteoclast activation and promoted the increase of bone trabeculae in the Bone metastases cancer. Trap is a typical marker of osteoclasts, and the results of Trap staining was that the cytoplasm of osteoclasts was wine red and the nucleus was light blue. Trap staining indicated that osteoclasts count was significantly increased in the Bone metastases group and was decreased after Bone metastases + Baicalin. HE staining is one of the commonly used staining methods in paraffin section technology. The nucleus is dyed purple blue and the cytoplasm is dyed pink. HE staining showed that the number of trabeculae decreased in the Bone metastases group and increased after Bone metastases + Baicalin.

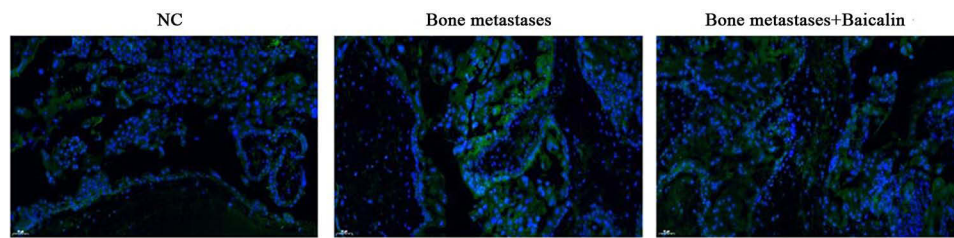


Figure 3 Baicalin inhibited the expression of iNOS in the Bone metastases cancer. iNOS was reported to be related with tumor metastasis. Fluorescein isothiocyanate (FITC) was labeled and showed bright yellow green fluorescence under fluorescence microscope. Immunofluorescence detection revealed that the expression of iNOS was significantly increased in the Bone metastases group and decreased after Bone metastases + Baicalin.

of iNOS was significantly increased in the Bone metastases group and decreased after Baicalin treatment (Figure 3).

Baicalin Inhibited the Expression of Inflammatory Factors in Bone Cancer Pain Rats

Subsequently, the protein level of inflammatory factors (IL-1 β , IL-6, TNF- α and PGE2) were examined in the three groups shown by ELISA. In Figure 4, the IL-1 β , IL-6, TNF- α and PGE2 protein level in the Bone metastases group was significantly higher than that of in the NC

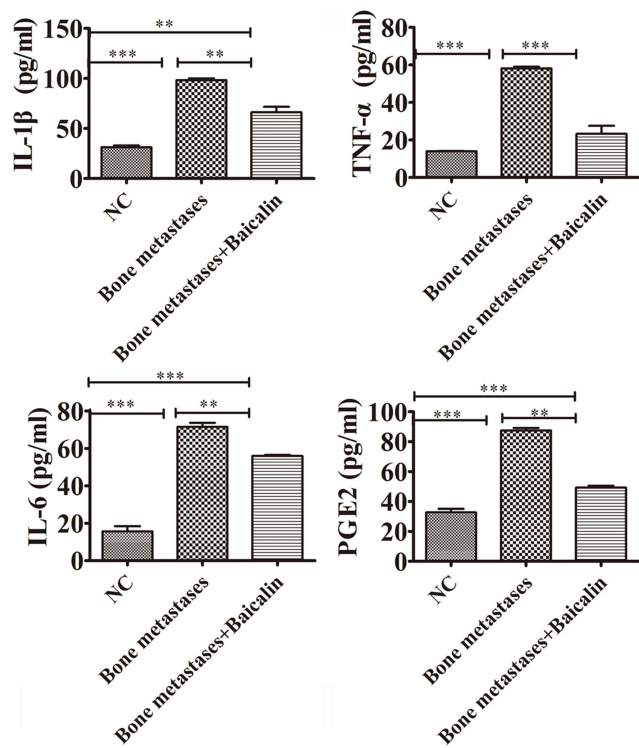


Figure 4 Baicalin inhibited the protein level of inflammatory factors (IL-1 β , IL-6, TNF- α and PGE2) in the Bone metastases cancer shown by ELISA. Data were shown as mean \pm SD. ** p < 0.01, *** p < 0.001 was obtained from the NC group vs the Bone metastases group, the NC group vs the Bone metastases + Baicalin group, the Bone metastases group vs the Bone metastases + Baicalin group.

group. After Baicalin treatment, IL-1 β , IL-6, TNF- α and PGE2 expression levels were significantly inhibited.

Transcriptome Sequencing Was Used to Detect the Therapeutic Effect of Baicalin on Bone Cancer Pain

In order to further study the therapeutic effect of Baicalin on bone cancer pain, we took the spinal cord tissues of SD rats in the NC group, the Bone metastases group and the Bone metastases + Baicalin group for transcriptome sequencing. The GO and KEGG enrichment analysis of DEGs were utilized to study the transcriptome differences in the Bone metastases group vs the NC group (Tables 1 and 2) and the Bone metastases + Baicalin group vs the NC group (Tables 3 and 4). In Tables 1 and 2, the results of top 10 enriched GO pathways of upregulated DEGs showed that the BP changes were in regulation of cellular process, regulation of nitrogen compound metabolic process, ribosome biogenesis, cell cycle, ncRNA processing, response to stimulus, rRNA metabolic process, rRNA processing, organelle organization and positive regulation of cellular metabolic process. Also, the CC changes of DEGs were obviously abundant in nucleus, nucleoplasm, nuclear lumen, intracellular membrane-bounded organelle, cytoplasm, cytosol, mitochondrion and mitochondrial membrane. Changes in MF were mainly enriched in protein binding, protein binding, enzyme binding, metal ion binding, cation binding, catalytic activity, nucleoside-triphosphatase activity and pyrophosphatase activity. The KEGG pathway analysis revealed that the upregulated DEGs were mainly enriched in ribosome biogenesis in eukaryotes, cell cycle, DNA replication, colorectal cancer, pyrimidine metabolism, mismatch repair, biosynthesis of amino acids, estrogen signaling pathway, alanine aspartate and glutamate metabolism, purine metabolism.

In the Tables 3 and 4, the results of top 10 enriched GO pathways of downregulated DEGs displayed that the BP

Table 1 Top 10 Enriched GO Pathways of DEGs Between the Bone Metastases Group and the NC Group

Terms	Pathway Description	Count	p-value
Upregulated			
GO.BP:0050794	Regulation of cellular process	411	2.09E-42
GO.BP:0051171	Regulation of nitrogen compound metabolic process	270	9.39E-41
GO.BP:0042254	Ribosome biogenesis	54	2.01E-34
GO.BP:0007049	Cell cycle	99	2.12E-29
GO.BP:0034470	ncRNA processing	55	6.03E-29
GO.BP:0050896	Response to stimulus	299	3.40E-28
GO.BP:0016072	rRNA metabolic process	43	3.82E-28
GO.BP:0006364	rRNA processing	42	7.49E-28
GO.BP:0006996	Organelle organization	169	5.24E-26
GO.BP:0031325	Positive regulation of cellular metabolic process	161	4.05E-25
GO.CC:0005654	Nucleoplasm	234	5.65E-52
GO.CC:0043227	Membrane-bounded organelle	554	3.70E-51
GO.CC:0005737	Cytoplasm	487	1.12E-50
GO.CC:0043231	Intracellular membrane-bounded organelle	521	6.67E-49
GO.CC:0005829	Cytosol	235	5.03E-34
GO.CC:0005739	Mitochondrion	115	8.29E-31
GO.CC:0005740	Mitochondrial_envelope	68	2.00E-23
GO.CC:0031981	Nuclear lumen	271	2.96E-22
GO.CC:0031966	Mitochondrial membrane	62	8.90E-21
GO.CC:0005634	Nucleus	378	2.01E-20
GO.MF:0005515	Protein binding	556	1.88E-76
GO.MF:0003824	Catalytic activity	251	5.28E-24
GO.MF:0042802	Identical protein binding	104	1.18E-15
GO.MF:0003677	DNA binding	118	2.89E-13
GO.MF:0019899	Enzyme binding	100	8.83E-10
GO.MF:0046872	Metal ion binding	158	8.90E-10
GO.MF:0043169	Cation binding	159	3.26E-09
GO.MF:0036094	Small molecule binding	107	4.37E-09
GO.MF:0017111	Nucleoside-triphosphatase activity	47	1.50E-08
GO.MF:0016462	Pyrophosphatase activity	49	1.92E-08
Downregulated			
GO.BP:0050794	Regulation of cellular process	547	3.74E-65
GO.BP:0050896	Response to stimulus	443	6.24E-64
GO.BP:0007154	Cell communication	312	5.02E-47
GO.BP:0007165	Signal transduction	294	8.60E-46
GO.BP:0023052	Signaling	306	1.36E-45
GO.BP:0010033	Response to organic substance	207	3.71E-41
GO.BP:0071310	Cellular response to organic substance	175	2.42E-38
GO.BP:0002376	Immune system process	189	7.67E-36
GO.BP:0032502	Developmental process	293	2.71E-33
GO.BP:0034097	Response to cytokine	107	3.91E-33
GO.CC:0016020	Membrane	498	5.88E-68
GO.CC:0005737	Cytoplasm	596	4.77E-52
GO.CC:0012505	Endomembrane_system	292	3.58E-50
GO.CC:0031982	Vesicle	270	1.08E-49
GO.CC:0031224	Intrinsic component of membrane	318	2.11E-45
GO.CC:0071944	Cell periphery	327	2.48E-44
GO.CC:0005886	Plasma membrane	317	1.46E-41

(Continued)

Table 1 (Continued).

Terms	Pathway Description	Count	p-value
GO.CC:0016021	Integral component of membrane	302	9.88E-41
GO.CC:0005576	Extracellular region	274	1.68E-39
GO.CC:0005615	Extracellular space	232	1.39E-38
GO.MF:0005515	Protein binding	680	1.57E-76
GO.MF:0003824	Catalytic activity	297	5.97E-22
GO.MF:0005102	Signaling receptor binding	116	4.58E-17
GO.MF:0042802	Identical protein binding	120	1.84E-14
GO.MF:0019899	Enzyme binding	132	1.81E-13
GO.MF:0016787	Hydrolase activity	144	5.36E-13
GO.MF:0030234	Enzyme regulator activity	78	6.73E-13
GO.MF:0046872	Metal ion binding	194	4.64E-10
GO.MF:0043169	Cation binding	198	4.73E-10
GO.MF:0022857	Transmembrane transporter activity	72	7.36E-10

Abbreviations: BP, biological process; CC, cellular component; MF, molecular function; DEGs, differentially expressed genes.

changes were in regulation of cellular process, regulation of nitrogen compound metabolic process, cell cycle, mitotic cell cycle, regulation of cell cycle, positive regulation of cellular metabolic process, regulation of RNA metabolic

process, regulation of nucleic acid-templated transcription, regulation of transcription, regulation of RNA biosynthetic process. CC changes were enriched in cytoplasm, cytosol, nucleoplasm, membrane, membrane-bounded organelle,

Table 2 Top 10 Enriched KEGG Pathways of DEGs Between the Bone Metastases Group and the NC Group

ID	Pathway Description	Count	p-value
Upregulated			
hsa03008	Ribosome biogenesis in eukaryotes	15	1.30E-05
hsa04110	Cell cycle	14	2.49E-04
hsa03030	DNA replication	7	3.50E-04
hsa05210	Colorectal cancer	11	3.90E-04
hsa00240	Pyrimidine metabolism	8	1.31E-03
hsa03430	Mismatch repair	5	1.48E-03
hsa01230	Biosynthesis of amino acids	9	2.06E-03
hsa04915	Estrogen signaling pathway	13	2.11E-03
hsa00250	Alanine aspartate and glutamate metabolism	6	2.49E-03
hsa00230	Purine metabolism	12	3.64E-03
Downregulated			
hsa04657	IL-17 signaling pathway	21	2.69E-09
hsa04668	TNF signaling pathway	21	7.18E-08
hsa05134	Legionellosis	14	3.61E-07
hsa05164	Influenza A	25	6.41E-07
hsa05133	Pertussis	13	6.34E-05
hsa05165	Human papillomavirus infection	32	1.37E-04
hsa05323	Rheumatoid arthritis	14	1.41E-04
hsa05145	Toxoplasmosis	15	3.11E-04
hsa04060	Cytokine-cytokine receptor interaction	28	4.84E-04
hsa04210	Apoptosis	16	8.62E-04

Abbreviation: DEGs, differentially expressed genes.

Table 3 Top 10 Enriched GO Pathways of DEGs Between the Baicalin Treatment Group and the NC Group

Terms	Pathway Description	Count	p-value
Upregulated			
GO.BP:0050794	Regulation of cellular process	167	1.21E-18
GO.BP:0050896	Response to stimulus	127	5.94E-15
GO.BP:0023051	Regulation of signaling	70	4.99E-13
GO.BP:0051171	Regulation of nitrogen compound metabolic process	96	1.49E-11
GO.BP:0009966	Regulation of signal transduction	62	1.83E-11
GO.BP:0009968	Negative regulation of signal transduction	37	6.02E-11
GO.BP:0009893	Positive regulation of metabolic process	72	2.40E-10
GO.BP:0032502	Developmental process	89	3.97E-10
GO.BP:0050790	Regulation of catalytic activity	50	5.13E-10
GO.BP:0007275	Multicellular organism development	79	7.73E-10
GO.CC:0016020	Membrane	149	3.23E-18
GO.CC:0005737	Cytoplasm	189	9.24E-18
GO.CC:0043227	Membrane-bounded organelle	210	9.24E-15
GO.CC:0012505	Endomembrane system	87	3.62E-14
GO.CC:0031982	Vesicle	75	1.29E-11
GO.CC:0043231	Intracellular membrane-bounded organelle	188	7.27E-11
GO.CC:0005576	Extracellular region	76	4.87E-09
GO.CC:0031090	Organelle membrane	62	1.90E-08
GO.CC:0071944	Cell periphery	86	4.09E-08
GO.CC:0099503	Secretory vesicle	28	4.14E-08
GO.MF:0005515	Protein_binding	236	3.12E-37
GO.MF:0003824	Catalytic_activity	105	2.71E-11
GO.MF:0042802	Identical_protein_binding	46	1.21E-08
GO.MF:0043169	Cation binding	68	2.81E-05
GO.MF:0004857	Enzyme inhibitor activity	14	2.85E-05
GO.MF:0016740	Transferase activity	43	4.70E-05
GO.MF:0046872	Metal ion binding	65	7.93E-05
GO.MF:0030234	Enzyme regulator activity	24	1.35E-04
GO.MF:0008427	Calcium-dependent protein kinase inhibitor activity	2	2.73E-04
GO.MF:0008454	Alpha-1,3-mannosylglycoprotein 4-beta-N-acetylglucosaminyltransferase activity	2	2.73E-04
Downregulated			
GO.BP:0050794	Regulation of cellular process	208	3.04E-28
GO.BP:0051171	Regulation of nitrogen compound metabolic process	121	5.28E-17
GO.BP:0007049	Cell cycle	49	4.65E-16
GO.BP:0000278	Mitotic cell cycle	35	1.28E-15
GO.BP:0051726	Regulation of cell cycle	45	1.70E-14
GO.BP:0031325	Positive regulation of cellular metabolic process	78	1.38E-13
GO.BP:0051252	Regulation of RNA metabolic process	84	3.03E-13
GO.BP:1903506	Regulation of_nucleic_acid-templated_transcription	80	3.24E-13
GO.BP:0006355	Regulation of transcription, DNA-templated	79	3.49E-13
GO.BP:2001141	Regulation of RNA biosynthetic_process	80	3.53E-13
GO.CC:0005737	Cytoplasm	223	5.24E-22
GO.CC:0005829	Cytosol	114	1.39E-18
GO.CC:0043227	Membrane-bounded organelle	242	5.54E-16
GO.CC:0005654	Nucleoplasm	87	2.43E-13
GO.CC:0043231	Intracellular membrane-bounded organelle	216	1.69E-11
GO.CC:0016020	Membrane	147	1.47E-10
GO.CC:0012505	Endomembrane system	86	6.92E-10

(Continued)

Table 3 (Continued).

Terms	Pathway Description	Count	p-value
GO.CC:0005856	Cytoskeleton	52	6.85E-09
GO.CC:0015630	Microtubule cytoskeleton	36	8.45E-09
GO.CC:0031090	Organelle membrane	68	4.64E-08
GO.MF:0005515	Protein binding	259	4.97E-35
GO.MF:0046872	Metal ion binding	82	1.24E-07
GO.MF:0043169	Cation binding	82	3.80E-07
GO.MF:0003824	Catalytic activity	98	7.76E-06
GO.MF:0140110	Transcription regulator activity	41	8.11E-05
GO.MF:0045294	Alpha-catenin binding	3	1.46E-04
GO.MF:0008092	Cytoskeletal protein binding	24	2.58E-04
GO.MF:0003677	DNA binding	47	2.64E-04
GO.MF:0030332	Cyclin binding	4	3.05E-04
GO.MF:0030234	Enzyme regulator activity	25	3.87E-04

Abbreviations: BP, biological process; CC, cellular component; MF, molecular function; DEGs, differentially expressed genes.

endomembrane system, cytoskeleton and organelle membrane. The MF changes were mainly enriched in protein binding, metal ion binding, cation binding, alpha-catenin binding, cytoskeletal protein binding, DNA binding, cyclin

binding, catalytic activity, transcription regulator activity and enzyme regulator activity. As for the top 10 KEGG KEGG pathway enrichment, the mRNAs were mainly enriched in AGE-RAGE signaling pathway in diabetic

Table 4 Top 10 Enriched KEGG Pathways of DEGs Between the Baicalin Treatment Group and the NC Group

ID	Pathway Description	Count	p-value
Upregulated			
hsa04640	Hematopoietic cell lineage	6	0.005
hsa05205	Proteoglycans in cancer	9	0.006
hsa05145	Toxoplasmosis	6	0.009
hsa04610	Complement and coagulation cascades	5	0.011
hsa01100	Metabolic pathways	32	0.045
hsa00270	Cysteine and methionine metabolism	3	0.046
hsa00330	Arginine and proline metabolism	3	0.046
hsa00510	N-Glycan biosynthesis	3	0.046
hsa05323	Rheumatoid arthritis	4	0.062
hsa00790	Folate biosynthesis	2	0.064
Downregulated			
hsa04933	AGE-RAGE signaling pathway in diabetic complications	7	0.001
hsa05168	Herpes simplex virus 1 infection	17	0.001
hsa05202	Transcriptional misregulation in cancer	9	0.003
hsa05132	Salmonella infection	10	0.005
hsa04064	NF-kappa B signaling pathway	6	0.005
hsa04144	Endocytosis	10	0.005
hsa05144	Malaria	4	0.007
hsa04668	TNF signaling pathway	6	0.007
hsa05020	Prion disease	10	0.009
hsa04218	Cellular senescence	7	0.010

Abbreviation: DEGs, differentially expressed genes.

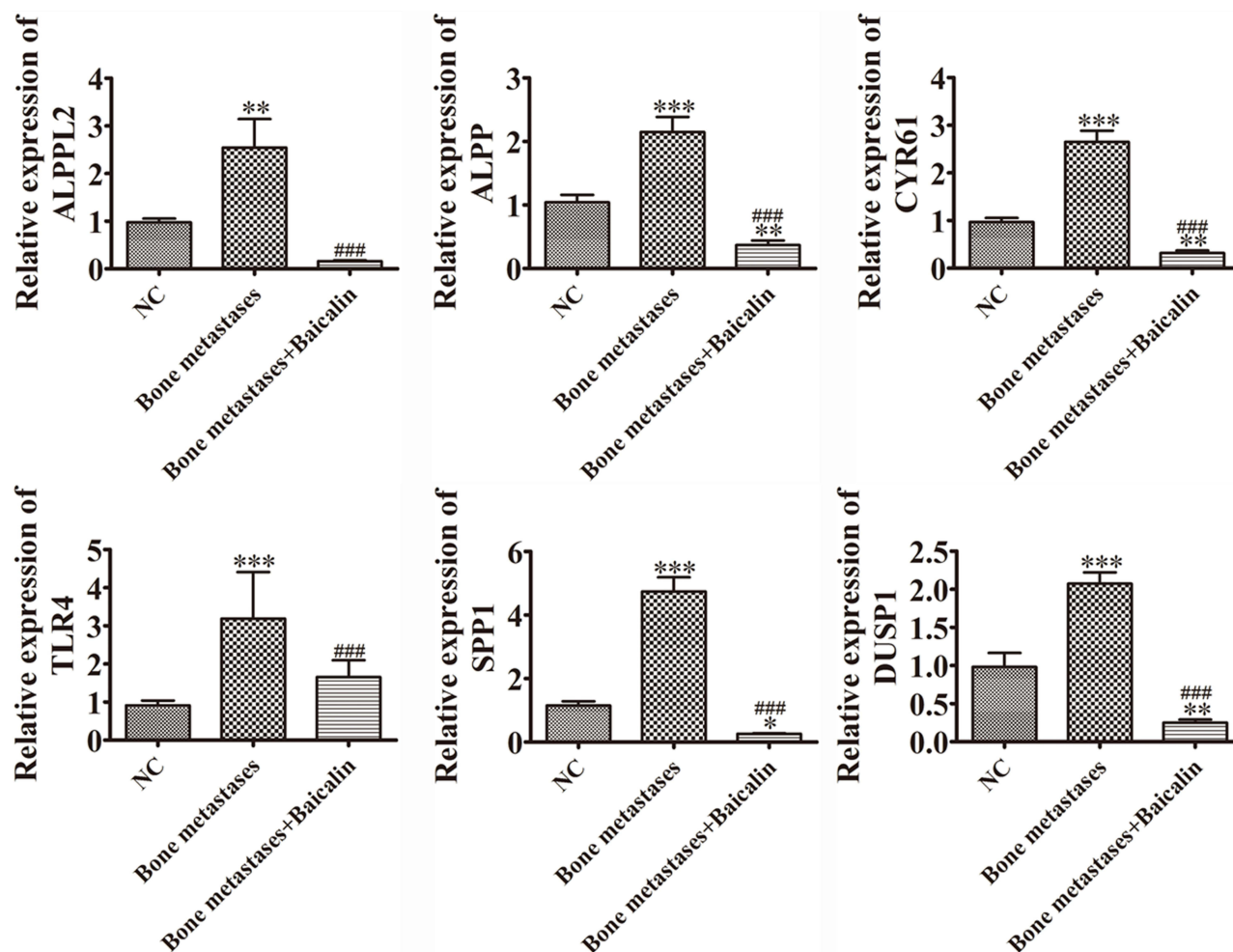


Figure 5 RT-qPCR results indicated that the mRNA expression level of *ALPP*, *DUSP1*, *CYR61*, *ALPPL2*, *SPP1* and *TLR4* were significantly up-regulated in the Bone metastases group compared with the NC group. By comparing the Bone metastases + Baicalin group and the NC group, the expression of these genes were significantly down-regulated. The RT-qPCR results of these genes were consistent with the results of the transcriptomic sequencing results. Data were shown as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ was obtained from the NC group vs the Bone metastases group or the Bone metastases + Baicalin group. ### $p < 0.001$ was obtained from the Bone metastases group vs the Bone metastases + Baicalin group.

complications, herpes simplex virus 1 infection, salmonella infection, prion disease, malaria, endocytosis, cellular senescence, transcriptional misregulation in cancer, NF-kappa B signaling pathway, TNF signaling pathway.

RT-qPCR Was Used to Verify the Sequencing Results

According to the transcriptomic sequencing results, the mRNA expression levels of *ALPP*, *DUSP1*, *CYR61*, *ALPPL2*, *SPP1* and *TLR4* were significantly up-regulated in the Bone metastases group compared with the NC group. By comparing the Bone metastases + Baicalin group and the NC group, the protein expression level of these genes was significantly down-regulated. The RT-

qPCR results of these genes were consistent with the results obtained by transcriptomic sequencing (Figure 5).

Discussion

It has been reported that the development of bone cancer pain was accompanied by the expression of many genes in peripheral and central nervous system were changed.^{16–19} Some researchers also found the changes of bone cancer pain gene expression profile by transcriptome sequencing. Some genes for the progression of pain hypersensitivity have also been discovered. However, the specific mechanism is still not very clear. In the study, we constructed the SD rat model of bone metastasis induced by MADB-106 cells and treated with baicalin. Based on the transcriptome

sequencing results and RT-qPCR validation experiments, we found that baicalin had a certain inhibitory effect on the SD rat model of bone metastasis cancer.

Natural products of plants were said to treat chronic diseases, such as pain.²⁰ Baicalin is a flavonoid extracted from Huang Qin, which has a variety of physiological functions. It has antioxidant properties,²¹ and has analgesic effect on migraine induced by nitroglycerin in rats,²² neuropathic pain in rats with spinal nerve ligation.^{23,24} Also, it was reported that it possessed anti-tumor effect in osteophilic breast cancer through inducing cell apoptosis.²⁵ However, the presumptive effect of baicalin on bone cancer pain and its underlying mechanism were not yet clear. In the latest research, Li et al²⁶ found that baicalin can improve mechanical hyperalgesia and thermal hyperalgesia in SD rats with bone cancer pain. In chicken liver inflammation induced by lipopolysaccharide, Cheng et al²⁷ reported that baicalin can inhibit the expression of iNOS by TLR4-Mediated NF- κ B Pathway. Min et al²⁸ revealed that baicalin suppressed the higher expression level of iNOS in the process of UVB-induced inflammatory injury by TLR4 pathway. The above results showed that baicalin may regress the expression of iNOS in the process of inflammatory response by TLR4 pathway or TLR4-mediated pathway. In this study, baicalin can inhibit the expression of iNOS in the SD rat model of bone metastasis cancer. In the follow-up, we still need to further explore what pathway baicalin plays a role. Additionally, the results showed that baicalin recovered the bone mineral density and the number of bone trabeculae in the SD rat with bone cancer pain, and inhibited the activation of osteoclasts. On the basis of the transcriptome sequencing results, the expression levels of some inflammatory cytokines were significantly decreased after baicalin treatment of bone cancer pain model. The results illustrated that baicalin had a certain inhibitory effect on the rat model of bone metastasis cancer. These insights can guide future research on the molecular mechanism of bone cancer pain, lay a theoretical foundation for new molecular markers related to pain response, and provide a theoretical basis for baicalin in the treatment of bone pain caused by breast cancer in the future.

Ethics Approval and Consent to Participate

All experimental procedures have been approved by the Ethics Committee of People's Hospital of Inner Mongolia (202021009L). Animal treatment was performed according to the guidelines of the International Association for pain research.²⁹

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Disclosure

The authors state that they have no conflicts of interest.

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