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Original Article

Effect of the Jianpi Bushen prescription on the expression profiles of genes associated with bone marrow hematopoiesis in radiation-damaged mice

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Abstract: The effects of the traditional Chinese drug Jianpi Bushen prescription (JBP) on the expression profiles of genes associated with bone marrow hematopoiesis in radiation-damaged mice were examined. A radiation damage model was induced in mice by single total body irradiation. Total RNA in bone marrow mononuclear cells of radiation-damaged mice was collected after treatment with JBP for 9 days. Gene expression profiles were detected using a whole-genome chip. The gene expression profile revealed 261 significantly different genes, 108 of which were up-regulated and 153 were down-regulated, in the radiation-damage model group as compared to those in the control group. High-dose (200%) and low-dose (100%) JBP reversed 35 and 42 differential genes, respectively, induced by radiation. Gene Ontology (GO) analysis showed that these reversed differentially regulated genes were related mainly to cell adhesion, material transport, cell growth, proliferation, differentiation, signal transduction, substance metabolism, immunity, and stimulation. Pathway analysis revealed that there were 34 differentially regulated genes in 10 signaling pathways related to hematopoietic processes, with the Wnt signaling pathway showing the best results. JBP improved bone marrow hematopoietic function in radiation-damaged mice by regulating multiple genes, functions, and signaling pathways.

Keywords: Jianpi Bushen prescription, radiation damage, gene expression profile, bone marrow hematopoietic, molecular mechanisms

Introduction

With advancements in technology and industry, radiation damage has become an important factor threatening human health. Radiation can damage of the immune system and bone marrow hematopoietic system, and can cause cancer [1, 2]. Cell growth factors might be used to counteract radiation damage [3]. However, the use of cell growth factors is expensive and shows some limitations. Furthermore, previous studies have shown that cell growth factors might lead to the continued survival of tumor cells and might counteract chemical drugs [4]. Therefore, it is important to identify safe, efficient, and low-toxicity drugs that can promote the growth of hematopoietic stem cells. Clinical trials have confirmed the effectiveness of traditional Chinese medicine in anti-radiation hematopoietic injury. The protective effect against hematopoietic system damage occurs through multiple pathways, such as promoting the regulation of hematopoietic cell proliferation and immune function [5, 6]. Jianpi Bushen prescription (JBP; previously known as Digan oral liquid) protects against radiation and chemical damage. Previous clinical trials have shown that JBP improves peripheral blood and enhances immunological function. Animal experiments further demonstrated the protective mechanisms of JBP against radiation damage with respect to marrow colony-stimulating factor, expression of apoptosis-related genes in the marrow and spleen, and splenic histopathological changes [7-9]. However, its mechanism remains unclear. Radiation alters gene expression in organisms through a complex signal transduction network [10]. In the present study, we applied genome-wide expression profile chip technology with bioinformatics analysis to identify genes and

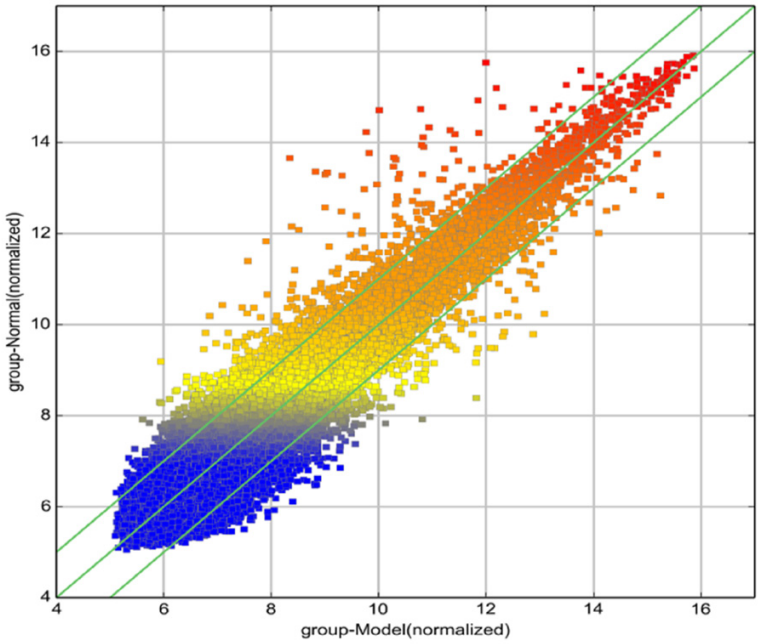

Figure 1. Effect of radiation damage on gene expression profile of bone narrow in radiation-damaged mice. Mice were treated with single total body irradiation (dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Radiation group were screened to 261 significantly differential genes, 108 out of which are up-regulated genes and 153 are down-regulated genes with a fold change 3 as the standard (The horizontal axis is the model group while the ertical axis is the nomal group).

key pathways affected by JBP following radiation damage, particularly bone marrow hematopoietic system damage. We then explored the hub genes and clarified the protective effect of JBP in the molecular mechanism of radiation damage. Our results provide a foundation for the clinical recovery of radiation-induced bone marrow hematopoietic damage.

Materials and methods

Animals

Sixty healthy male and female Kunming mice weighing 18-22 g were used in this study. These mice were provided by the Hubei Province Experimental Animal Research Center. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan General Hospital of Guangzhou Military Area Command.

Drug preparation

JBP was prepared from a large dose of prepared rehmannia root and honey-fried licorice root, supplemented with angelica, radix astragalus, and tangerine peel. The prescription was prepared by the Department of Pharmaceutical Preparation in the Wuhan General Hospital of Guangzhou Military Region Hospital. The extract liquid of the prescription was subjected to water decoction and filtration. Next, the extract liquid of the prescription was concentrated to 100% (1 g crude drug/mL) and 200% (2 g crude drug/mL) liquid medicine. This liquid was then packaged and stored.

Main reagents and apparatus

The RNAprep pure animal total RNA extraction kit was purchased from the Beijing TianGen Biochemical Company (Beijing, China). The Superscript ds-cDNA short kit was from Invitrogen (Carlsbad, CA, USA). The NimbleGen One Color Kit, DNA Labeling NimbleGen Hybridization Kit, and NimbleGen Wash Buffer Kit were all purchased from Roche NimbleGen (Basel, Switzerland). NimbleScan software (version 2.5) was from Roche. The NanoDrop ND-1000 was from Thermo Scientific (Waltham, MA, USA). The NimbleGen Hybridization system and GenePix Pro 6.0 software were purchased from Roche NimbleGen. The Microarray GenePix 4000b scanner was from Molecular Devices Corporation (Sunnyvale, CA, USA). Agilent GeneSpring GX software was from Agilent Corporation (Santa Clara, CA, USA).

Animals and grouping

Sixty male Kunming mice (18-22 g) were obtained from the Hubei Province Experimental Animal Research Center. Fifteen mice were randomly selected in the normal control group, and the remaining 45 mice were treated with single total body irradiation (dose: 6.0 Gy, dose

Table 1. Gene Ontology types of differential genes induced by radiation

GO Name	Gene Numbe	
	Up	Down
Molecular_function		
Binding	224	301
Catalytic activity	0	142
Transporter activity	0	0
Transferase activity	0	51
Enzyme regulator activity	26	25
Structural molecule activity	0	0
Electron carrier activity	0	0
Antioxidant activity	0	0
Cellular_component		
Organelle	184	257
Cell part	255	362
Extracellular region	0	0
Envelope	17	22
Membrane-enclosed lumen	0	56
Biological_process		
Cellular process	239	334
Metabolic process	175	265
Localization	86	0
Death	0	0
Locomotion	30	30
Growth	0	0
Pigmentation	0	0
Canonical Wnt receptor signaling pathway	75	

Mice were treated with single total body irradiation (dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Radiation group were screened to 261 significantly differential genes, 108 out of which are up-regulated genes and 153 are down-regulated genes. Differential genes related to cell adhesion, transport, cell growth, proliferation, differentiation, signal transduction, metabolism, immunity and stimulating and so on.

rate: 450 Gy/min) using the linear accelerator X line for 9 days to prepare the radiation damage mice model. Decreased appetites and reduced activity levels were used as criteria for successful modeling of mice; compared with that of the normal group, the white blood cell count was significantly reduced in the radiation damage model mice (normal group: 12.61×10^9/L, radiation-damaged mice: 7.34×10^9/L). According to the random number table, 45 mice were divided into the model group, low-dose JBP group (100% concentration), and high-dose JBP group (200% concentration). Normal control and model group mice were administered 0.2 mg/10 g sodium chloride by lavage twice per day. The other two groups were respectively administered 0.1 g/kg suspension low-dose JBP or high-dose JBP by lavage twice per day. The mice were sacrificed on the ninth day, and the bilateral femur and tibia bone marrow were collected.

Isolation and in vitro culture of bone marrow cells from mice

Under aseptic conditions, after 9 days mice in the experimental group were sacrificed by breaking the neck followed by 75% alcohol immersion for 3-5 min. The muscle was carefully removed, cutting off the cartilage on both ends, to reveal the red marrow cavity. The marrow cavity of the cell was washed with 2.5 ml PBS for each bone. This solution was centrifuged and the supernatant was removed. After resuspending the cells, the cells were lysed in ammonium chloride solution, centrifuged at 1200×g revolutions for 10 min and the supernatant was removed. Finally, the bone marrow cells were frozen in liquid nitrogen until use.

RNA extraction and purification

The chips, which were tested by Wuhan Baffil Biological Technology Co., Ltd., were mice bone marrow expression profile chips (Roche NimbleGen, USA). The general process was as follows: total RNA was extracted from bone marrow cells using an RNA extraction kit; the integrity and concentration of RNA was evaluated; reverse transcription of cDNA was conducted and the samples were purified. Purified cDNA was hybridized to Cy3 and the chip at 42°C overnight.

Chip test and statistical analysis

Images were acquired using the GenePix 4000B scanner (Molecular Devices) controlled by GenePix Pro6.0 with NimbleScan v2.5 software. All data were further analyzed using Agilent GeneSpring GX v12.1 software. We first selected high-quality signals based on the expression value and then screened for differentially expressed genes between two samples in a fold-change or *t*-test. R script was used for cluster analysis, and then the standard concen-

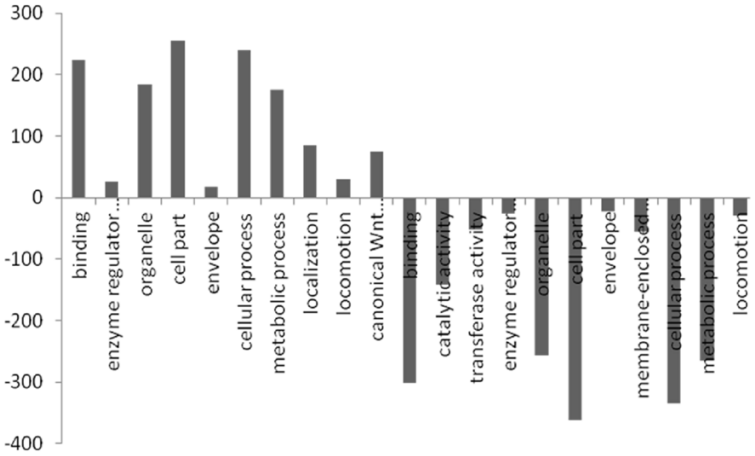

Figure 2. Gene Ontology types of differential genes induced by radiation. Mice were treated with single total body irradiation (dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Radiation group were screened to 261 significantly differential genes, 108 out of which are up-regulated genes and 153 are down-regulated genes. Differential genes related to cell adhesion, transport, cell growth, proliferation, differentiation, signal transduction, metabolism, immunity and stimulating and so on (The positive axis is the up-regulated gene ontology types while the negative axis is the down-regulated gene ontology types).

Table 2. Hematopoietic relative pathway category of differential genes induced by radiation

Pathway Category	Pathway Number	
	Up	Down
Regulation of actin cytoskeleton	9	0
MAPK signaling pathway	10	0
Hematopoietic cell lineage	0	0
Rap1 signaling pathway	0	0
Cell adhesion molecules	0	0
p53 signaling pathway	5	0
Pathways in cancer	11	17
PI3K-Akt signaling pathway	20	0
NF-kappa B signaling pathway	0	9
TNF signaling pathway	0	0
Cell cycle	8	9
Chronic myeloid leukemia	5	6
T cell receptor signaling pathway	0	0
Wnt signaling pathway	60	44

Mice were treated with single total body irradiation (dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Radiation group were screened to 261 significantly differential genes, 108 out of which are up-regulated genes and 153 are down-regulated genes. These differential genes were involved in fourteen signaling pathways associated with hematopoietic, with the Wnt signaling pathway producing the best results.

tration analysis method was used for GO and pathway analysis.

Results

Part 1: Radiation damage lead to the differences of gene expression

The Cy3 single standard chip used for hybridization contains 44,170 genes, and the original data has been uploaded to the Gene Exprssion Ominibus (GEO) database (No. GPL15887). The scatter diagram (**Figure 1**) shows the different gene distributions between the experimental group and model group. Each point in the figure represents gene expression. Genes showing lower expression value are located in the lower left corner, whereas those showing high expression are located in the upper right corner. Points above the 45° line indicate up-regulated gene expression, whereas those below this line indicate down-regulated gene expression. Greater deviation from the 45° line indicates greater changes in expression. The radiation group showed 261 significantly differentially expressed genes, 108 of which were up-regulated genes and 153 were down-regulated, based on a fold-change of 3 as the standard.

Gene ontology of differentially expressed genes

As shown in **Table 1** and **Figure 2**, differentially expressed genes were involved in cell adhesion, transport, cell growth, proliferation, differentiation, signal transduction, metabolism, immunity, and stimulation.

Hematopoietic relative pathway category of differentially expressed genes

There were 92 differentially expressed genes involved in 14 signaling pathways associated with hematopoiesis (**Table 2**; **Figure 3**), among which 32 genes were involved multiple pathways changes at the same time. Radiation

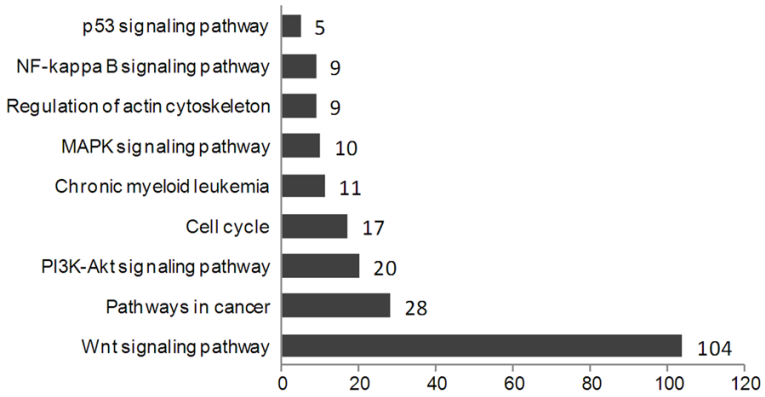

Figure 3. Hematopoietic relative pathway category of differential genes induced by radiation. Mice were treated with single total body irradiation (dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Radiation group were screened to 261 significantly differential genes, 108 out of which are up-regulated genes and 153 are down-regulated genes. These differential genes were involved in fourteen signaling pathways associated with hematopoietic, with the Wnt signaling pathway producing the best results (The horizontal axis is the differential gene numbers while the ertical axis is the pathway category).

change the expression of genes in different functions and systems, obviously in the hematopoietic system.

Part 2: JBP regulates the differentially expressed genes in radiation-damaged mice

The gene expression profile showed that there were 261 significantly differentially expressed genes, 108 of which were up-regulated and 153 were down-regulated, in the radiation-damage model group compared to those in the control group. Treatment with high-dose (200%) JBP group and low-dose (100%) JBP reversed the expression changes in 35 and 42 genes, respectively.

Gene ontology types of differentially expressed genes

As shown in **Table 3** and **Figure 4**, compared to those in the radiation group, the differentially expressed genes were related mainly to cell adhesion, material transport, cell growth, proliferation, differentiation, signal transduction, metabolism, immunity, stimulation, and other function.

Pathway analysis of differentially expressed genes

Compared to those in the radiation group, 34 differentially expressed genes affected by JBP were involved in hematopoiesis in 10 signal pathways (**Table 4**; **Figure 5**). JBP mitigates the effects of radiation on the body, especially in the hematopoietic system.

Discussion

Radiation damage is categorized as a consumptive disease or syndrome causing blood deficiency. Therapy typically involves treating the deficiency with tonification in traditional Chinese medicine. JBP is an empirical formulation prepared in our department, which is composed of *Radix rehmanniae preparata*, honey-fried licorice root, Chinese angelica, astragalus, tangerine peel, and nourish qi to affect the spleen and kidney. Recent pharmacological studies have reported that rehmannia root preparations can increase immunity and promote hematopoietic stem cell proliferation [11, 12]. Angelica polysaccharide significantly promoted the proliferation and differentiation of hematopoietic cells, particularly by reducing peripheral blood cells and bone marrow suppression [13, 14], and by regulating the hematopoietic microenvironment to promote the proliferation of hematopoietic cells [15]. Astragalus can significantly improve the peripheral blood cell level, promote secretion of hematopoietic cytokines, stimulate the function of the hematopoietic system, and improve the bone marrow suppression state induced by chemotherapy and radiotherapy [16, 17]. In summary, JBP can promote hematopoietic cells and immune adjustment functions.

We evaluated the effects of radiation on the gene expression profiles of mouse using a whole-genome chip. We detected 261 significant differentially expressed genes, which included 108 upregulated and 153 downregulated genes. GO function analysis revealed differences in genes associated with cell adhesion, material transport, cell growth, proliferation and differentiation, signal transduction, metabolism, immunity, and stimulation, among other functions. Pathway analysis revealed 92 differen-

Table 3. Gene Ontology types of differential genes interfered by Jianpi Bushen Prescription (JBP) in radiation-damaged mice

GO Name	Gene Number		Gene name	
	Up	Down	Up	Down
Molecular_function				
Binding	8	19	FGFR2, NR4A1, PIK3CD, BCL2L1	MAPK1, MAPK8, WNT5A, PPP3R1
Catalytic activity	7	13	CDC25B, PIK3CD, CAMK2A, CAMK2B	PLCB4, CHD8, CSNK2A1, CSNK1E
Transferase activity		9		MAPK1, ATF2, NLK, MAPK8
Enzyme regulator activity	1	6	BCL2L1, CSNK2B	AR, HSPB1, RASGRP3, LRP6
Structural molecule activity		1		AR
Electron carrier activity		1		AR
Cellular_component				
Organelle	8	15	CDC25B, FGFR2, NR4A1, BCL2L1	MAPK1, YWHAZ, SENP2, SMAD4
Cell part	9	18	CDC25B, FGFR2, NR4A1, PIK3CD	ITGA6, ITGB1, SFRP4, WNT5A
Extracellular region	2	8	FGFR2, CSNK2B,	ITGA6, ITGB1, HSPB1, WNT5A

Mice were treated with single total body irradiation(dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Mice were administered lavage with 0.1 g/kg suspension low-dose JBP (100%), high-dose JBP (200%) twice a day for 9 days. It respectively reverse 35, 42 differential genes induced by radiation after intervening with the high-dose (200%) JBP group and low-dose (100%) JBP group. Differential genes related to cell adhesion, material transport, cell growth, proliferation, differentiation, signal transduction, metabolism, immunity and stimulating, and other aspects.

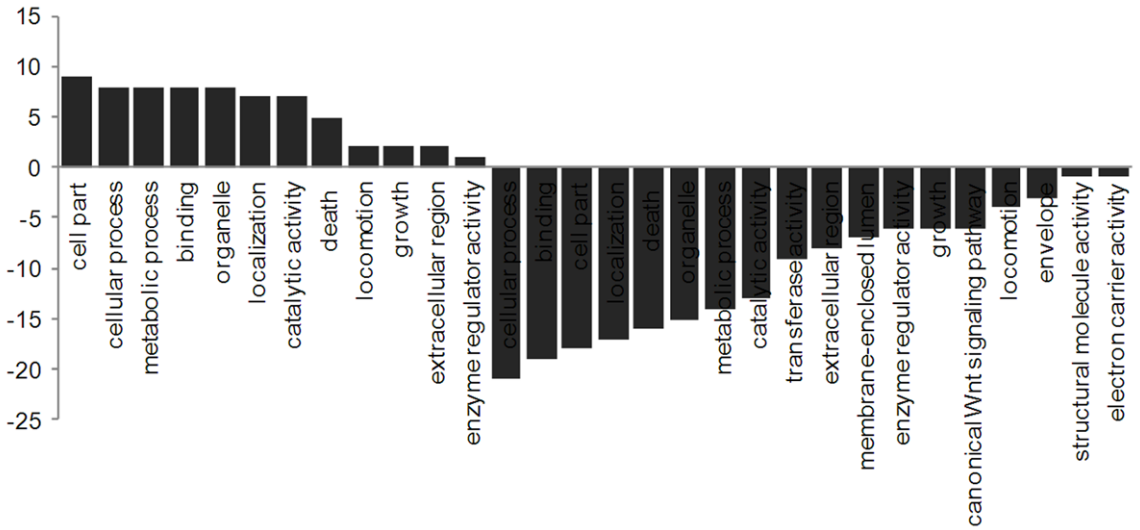

Figure 4. Gene Ontology types of differential genes interfered by Jianpi Bushen Prescription (JBP) in radiation-damaged mice. Mice were treated with single total body irradiation (dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Mice were administered lavage with 0.1 g/kg suspension low-dose JBP (100%), high-dose JBP (200%) twice a day for 9 days. It respectively reverse 35, 42 differential genes induced by radiation after intervening with the high-dose (200%) JBP group and low-dose (100%) JBP group. Differential genes related to cell adhesion, material transport, cell growth, proliferation, differentiation, signal transduction, metabolism, immunity and stimulating, and other aspects (The positive axis is the up-regulated gene ontology types while the negative axis is the down-regulated gene ontology types).

tially expressed genes involved in hematopoietic signaling pathways, including 14 genes related to the Wnt signaling pathway. The results verify the effect of ionizing radiation on cell injury with multi-target, multi-level, and multi-channel characteristics. After treatment with different doses of JBP, differentially expressed genes were reduced, suggesting that JBP mitigates the effects of radiation on the body.

The high-dose and low-dose groups showed reversed expression of 35 and 42 radiation-induced genes, respectively. These genes were mainly involved in cell adhesion, material trans-

Table 4. Hematopoietic relative pathway category of differential genes interfered by Jianpi Bushen Prescription (JBP) in radiation-damaged mice

Pathway Category	Gene Number		Gene name	
	Up	Down	Up	Down
MAPK signaling pathway	3		CDC25B, FGFR2, NR4A1	
Rap1 signaling pathway	2		FGFR2, PIK3CD	
NF-kappa B signaling pathway	1		BCL2L1	
T cell receptor signaling pathway		1		MAPK1
Chronic myeloid leukemia		1		MAPK1
Pathways in cancer		4		MAPK1, AR, ITGA6, ITGB1
TNF signaling pathway		2		MAPK1, ATF2
MAPK signaling pathway		5		MAPK1, HSPB1, IL1A, NLK
Cell cycle		1		YWHAZ
Wnt signaling pathway	4	16	CAMK2A, CAMK2B, CSNK2B, PLCB3	LRP6, MAPK8, NLK, PLCB4, SFRP4, WNT5A, PPP3R1

Mice were treated with single total body irradiation(dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Mice were administered lavage with 0.1 g/kg suspension low-dose JBP (100%), high-dose JBP (200%) twice a day for 9 days. It can respectively reverse 35, 42 differential genes induced by radiation after intervening with the high-dose (200%) JBP group and low-dose(100%) JBP group. These differential genes were involved in ten signaling pathways associated with hematopoietic, with the Wnt signaling pathway producing the best results.

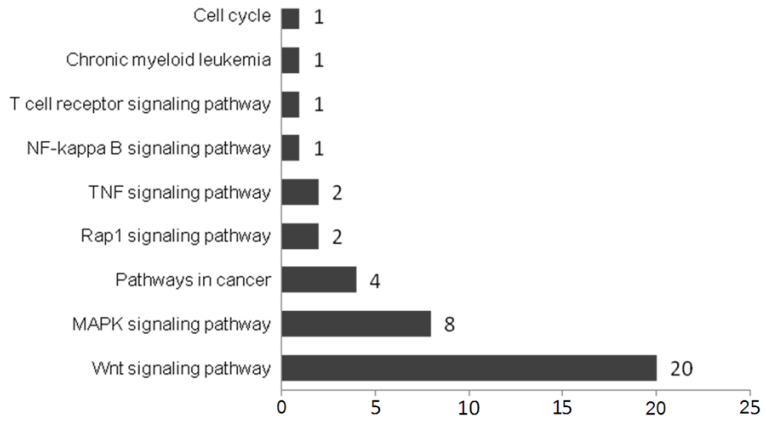

Figure 5. Hematopoietic relative pathway category of differential genes interfered by Jianpi Bushen Prescription (JBP) in radiation-damaged mice. Mice were treated with single total body irradiation (dose: 6.0 Gy, dose rate: 450 Gy/min) by using linear accelerator X line for 9 days to induce radiation damage mice model. Mice were administered lavage with 0.1 g/kg suspension low-dose JBP (100%), high-dose JBP (200%) twice a day for 9 days. It can respectively reverse 35, 42 differential genes induced by radiation after intervening with the high-dose (200%) JBP group and low-dose (100%) JBP group. These differential genes were involved in ten signaling pathways associated with hematopoietic, with the Wnt signaling pathway producing the best results (The horizontal axis is the differential gene numbers while the ertical axis is the pathway category).

port, cell growth, proliferation, differentiation, signal transduction, metabolism, immunity, and stimulation. Based on pathway enrichment, there 34 genes were associated with 10 hematopoietic pathways, among which the Wnt signaling pathway showed the most significant differences in gene expression.

The Wnt signaling pathway is a classic signaling pathway involved in cell growth and differentiation. Following binding to the frizzled protein family on the cell surface by low-density lipoprotein receptor-related protein, Wnt protein inhibits the activity of glycogen synthase kinase 3β, resulting in β-catenin dephosphorylation and accumulation in the cytoplasm. β-Catenin is then transferred to the nucleus and the TCF/LEF family of transcription factors forms a complex to regulate the transcription of cyclin D1, peroxisome proliferator-activated receptor γ (PPAR-γ), and other downstream target genes [18]. Cyclin D1 regulates the rate of cell proliferation in the cell cycle [19]. PPAR-γ is a member of the superfamily of nuclear hormone receptors and is an important transcription factor that induces adipocyte-specific gene expression and regulates fat cell differentiation [20]. The expression levels of PPAR-γ can be used as a specific marker of fat cell differentiation [21]. JBP promote hematopoietic cell proliferation and the self-renewal of hematopoietic cells, inhibits bone marrow fat, reduces the marrow fat vacuoles area, and improves the hematopoietic microenvironment by significantly increasing cyclin D1 levels and reducing PPAR-γ levels. This suggests that JBP may regulate the

expression of Wnt signaling pathways to promote bone marrow hematopoietic function.

In summary, JBP may significantly improve the hematopoietic system of radiation-injured mice. Gene microarray analysis showed that JBP has protective effects on the hematopoietic system through multi-gene, multi-functional, and multi-signaling pathways, such as by regulating molecules related to signaling pathways involved in cell proliferation, apoptosis, and cell differentiation to decrease hematopoietic system damage. Further studies are needed to combine traditional Chinese medicine and pharmacology network technology to explore the molecular effects of JBP on irradiation injury and determine the effectiveness of JBP.

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Disclosure of conflict of interest

None.

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