Study on Acupuncture and Moxibustion of the Notch Signaling Pathway in Bone Marrow Cells to Alleviate Cyclophosphamide-Induced Myelosuppression in Mice

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Abstract

Objective: To evaluate the protein expression and distribution of Notch signaling-related, differentially expressed genes (DEGs) in the bone marrow cells of mice. Additionally, verification of acupuncture regulated protein expression of these DEGs to protect bone marrow stem cells and alleviate cyclophosphamide (CTX) chemotherapy-induced myelosuppression was examined as well.

Methods: Thirty two Healthy male Kunming mice of clean grade were randomized into blank group (A), model group (B), acupuncture group (C) and moxibustion group (D). Mice in groups B, C, and D were intraperitoneally injected with 100mg/kg/d CTX for three successive days to generate the myelosuppressive model; while mice in group A were injected with an equivalent volume of sterile saline. After immobilizing the animals, mice in groups C and D received daily acupuncture and moxibustion in points "DU14", "BL17" "BL23" and " ST36" respectively,while those in group A and B were received no treatment. After five days of treatment, the mice were euthanized by cervical dislocation and their femurs collected for marrow harvest. Small-throughput cDNA microarray was used for initial DEG screening, followed by immunohistochemistry, real-time quantitative PCR (RT-qPCR), and Western Blot analysis to detect protein expression and quantity of the Notch signaling-related DEGs in the bone marrow cells.

Results: Expression of numb proteins (i.e., numb1 and numb2) was found to be upregulated while notch2 and jag1 were down regulated in bone marrow cells of acupuncture-treated, tumor-bearing mice.

Conclusion: Notch signaling pathway serves as an important signal transduction pathway for acupuncture and moxibustion to relieve CTX chemotherapy-induced myelosuppression. Regulation of associated DEGs in notch signaling pathway in marrow cells is the key mechanism by which acupuncture and moxibustion improve marrow hematopoiesis after chemotherapy.

Keywords: Acupuncture and Moxibustion; Chemotherapy; Myelosuppression; Notch signaling pathway

Abbreviations

CTX: Cyclophosphamide; DU14: Dazhui acupoint; BL17: Geshu acupoint; BL23: Shenshu acupoint; ST36: Zusanli acupoints.

Background

There is a significant increase in the incidence of cancer year by year on a global scale, which is estimated to reach 24 million by 2035. In Europe and the United States the incidence is 300-350/100000, with mortality rate 40%, while in China the incidence is 285.91/100000, with mortality rate as high as 80% [1,2].

Chemotherapy is one of the primary treatments for malignant tumors, however chemotherapeutics would induce varying degrees of damage to all systems of the body. Myelosuppression, as the most common and severe toxicity in the blood system, could reduce peripheral blood cells, resulting in compromised immunity, decreased body resistance and even infection, which may lead to the death of the patients and failure of the chemotherapy [3-4]. Main treatments of myelosuppression in modern medicine mainly include: injection of hematopoietic colony-stimulating factors, taking oral drugs to promote leucocytes, composition blood transfusion, marrow transplantation, etc. But these methods could easily relapse or rebound after treatment and the long-term curative effect is unstable [5]. Therefore, it is imperative to investigate the pathogenesis of post-chemotherapy myelosuppression and explore effective preventive measures, so as to reduce the toxicity of chemotherapy.

Preliminary study has demonstrated that: acupuncture and moxibustion could promote peripheral leucocytes, simulate recovery of pathologic DNA, accelerate the division and proliferation of marrow hematopoietic cells and relieve myelosuppression after chemotherapy, enhance hematopoiesis and immunity, thus improve the clinical symptoms and living quality of the patents. However, it will take a complex pathological restorative process to relieve myelosuppression by acupuncture and moxibustion, and the fundamental mechanism still requires further investigation. Cell signaling pathways involvement and regulation have become a hot topic for current researches.

Notch signaling pathway plays an important role in myelosuppression restoration and is capable of adjusting various stages of hematopoietic cells [6-7]. Notch receptors and ligands are highly expressed in hematopoietic progenitor cells and marrow stromal cells [8]. Notch signaling pathway could regulate hematopoietic stem/ progenitor cells by increasing self-renewal ability and relatively lowering differentiation capacity of the cells, so as to maintain stability of the stem cell pool [9].

This study adopts the CTX-induced myelosuppression modeling, after acupuncture and moxibustion treatment, screen out DEGs using gene chip technology, and then test the protein level and mRNA transcription of the DEGs in the Notch signaling pathway with immunohistochemistry, RT-PCR and Western Blot methods. Through analysis of the effect of acupuncture and moxibustion on associated DEGs of the Notch signaling, this study reveals the molecular biological mechanisms of acupuncture and moxibustion in relieving CTX chemotherapy-induced myelosuppression from the perspective of signal transduction pathway.
Materials and Methods

Experimental animals

32 male Kunming (KM) mice (aged 7 weeks, weighing 18 ± 2g) of clean grade were provided by Laboratory Animal Center of Henan Province (Zhejiang University School of Medicine). The mice were modeled after three days of free diets in the experimental environment with temperature 20~25 °C and relative humidity 60%. This study was approved by Ethics Committee of Henan University of Chinese Medicine. All procedures were in strict accordance with the guidelines for the care and use of laboratory animals formulated by Ministry of Science and Technology, People’s Republic of China [10].

Replication and Grouping of Myelosuppression Models

Thirty two male SPF Kunming (KM) mice were randomized into blank group (A), model group (B), acupuncture group (C) and moxibustion group (D), with eight mice in each group, kept in separate cages, four mice per cage. Modeling methods: Mice in group B,C,D were prepared into myelosuppression model with intraperitoneal injection of CTX at 100 mg/kg/d (Batch No: 04120501;0.2g/ml, PUDF Pharmaceutical Co. Ltd. Shanxi, China), for three successive days. The model was successfully established four hours after the last injection (Pharmacological activity disappears after 4 hours according to Cyclophosphamide pharmacokinetics [11]). Mice in group A were injected with the same dose of physiological saline (0.9% NaCl 250ml/ bottle; Shandong CISEN pharmaceutical Co., Ltd., Batch No: 120412107).

Acupoints Selection

According to the acupoints location methods established in Chinese Veterinary Acupuncture and Moxibustion, “DU14”: located in the center of the back, between the 7th cervical vertebra and 1st thoracic vertebrae; “BL17”: located on both side under the seventh thoracic; “BL23”: located between the rib under the second lumbar vertebra; “ST36”: located at the posterior-lateral of the leg knee joint, about 5 mm below the capitula fibula.

Treatments

Group A: Fixed but no treatment was given

Group B: Fixed but no treatment was given

Group C: Perpendicular insertion with mill-needle in DU14, BL17(double), BL23 (double), ST36(double), 6 min per point, with depth of 3 mm, once a day for 5 days [12], perpendicular insertion, apply reinforcing method of twirling technique for 5 times, twisting angle 90°, 200 times/min. (Jiangsu Wuxi jialian filliform needle, Specification: 0.19 cm × 10 mm, handle 20 mm)

Group D: Suspend moxibustion in DU14,BL17 (double), BL23 (double), ST36 (double), 3 min per point, with depth of 2 cm, once a day for 5 days[12-14] (Henan Nanyang Moxibustion products Co., Ltd., Specification: 0.4 cm × 25 cm)

Sample Collection

After five days of treatment, execute the mice by cervical vertebra luxation, get bilateral humerus and femurs on dry ice on a super-clean bench, remove all the muscles and connective tissues, flush with 0.9% saline, and then put them in -80 °C cryogenic refrigerator for inspection.

Statistical analysis

SPSS16.0 statistical software (SPSS, Chicago, IL) was used for data analysis. Data presented represent the mean (X) ± standard deviation (s) and statistical analysis was performed using ANOVA according to the standard α = 0.05. Levene’s test was used to access the homogeneity of variances. If the variance between populations was equal, LDS test was used for pairwise comparison; if the variance between populations was unequal, Tamhane’s test was used for pairwise comparison. P value < 0.05 was considered as statistically significant.

Results

Initial screening of the Notch signaling-related DEGs in mouse bone marrow cells in different groups using small-throughput microarray

Five days post-treatments four animals from each group were randomly selected for DEG screening using the Illumina iScan microarray platform (Genergy Bio-technology, Shanghai, China; MouseWG-6 whole-genome expression profiling BeadChips and reagents were provided by Illumina). Seven genes of the Notch signaling pathway, including notch1, notch2, jag1, jag2, delta1, numb1, and numb2 were found among the genes that showed differential expression in the bone marrow cells of mice in the four experimental groups.

Further test the Notch signaling-related DEGs in bone marrow hematopoietic cells with immunohistochemical method

Apply immunohistochemical method (Secondary antibodies, BSA, DAB concentrated kits, neutral resin, PBS fluid, IMS image analysis system, etc. Zhengzhou Huayu bio-technology Co., Ltd., parafin slicing machine, spreading machine, PPTHK-21 B, Laike Company) to test associated DEGs in notch signaling pathway in marrow hematopoietic cells after the preliminary screening.

Testing and analysis methods

Collected and analyze relevant parts of the samples by taking microscope pictures, calculate positive area and OD value. Use ImageJ2x software to calculate the positive area. Five horizons were collected from each film randomly. The average value of the positive areas in the five horizons would represent the index’s the level of expression. OD value was calculated with software.

<table>
<thead>
<tr>
<th>Differential gene</th>
<th>Sample size</th>
<th>Blank group</th>
<th>Acupuncture group</th>
<th>Moxibustion group</th>
</tr>
</thead>
<tbody>
<tr>
<td>delta1</td>
<td>8</td>
<td>711.38±1.269</td>
<td>739.50±52.331</td>
<td>725.13±46.912</td>
</tr>
<tr>
<td>notch1</td>
<td>8</td>
<td>788.25±8.4758</td>
<td>804.88±7.3480</td>
<td>807.88±6.3106</td>
</tr>
<tr>
<td>notch2</td>
<td>8</td>
<td>803.50±5.792</td>
<td>860.75±40.063</td>
<td>749.63±44.600</td>
</tr>
<tr>
<td>jag1</td>
<td>8</td>
<td>758.38±17.312</td>
<td>804.25±39.967</td>
<td>753.25±26.435</td>
</tr>
<tr>
<td>jag2</td>
<td>8</td>
<td>801.33±9.336</td>
<td>803.88±8.089</td>
<td>686.25±4.109</td>
</tr>
<tr>
<td>numb1</td>
<td>8</td>
<td>831.38±24.686</td>
<td>748.25±37.040</td>
<td>792.85±56.992</td>
</tr>
<tr>
<td>numb2</td>
<td>8</td>
<td>796.13±52.283</td>
<td>727.63±47.764</td>
<td>790.25±36.503</td>
</tr>
</tbody>
</table>

Table 1: The effect of acupuncture and moxibustion on DEGs protein expression in notch signaling pathway in CTX mice (x ±s,n=8)

Note: 1) compared with the blank group (P < 0.05), 2) compared with model group (P < 0.05) 3) compared with acupuncture group (P < 0.05).
The results indicated that 4 of the 7 DEGs screened out by gene expression profile had significant differences. The results are shown in table 1.

Results: On the basis of preliminary screening of associated DEGs with gene expression profiles, testing results had indicated that, after five days of treatment, notch2, jag1 and jag2 decreased while numb1 and numb2 raised in the model group, showing significant difference as compared with that in the blank group (P < 0.05).

Delta1 and notch1 had no statistical significance (P > 0.05); notch2, jag1 and jag2 decreased, numb1 and numb2 increased in acupuncture group and moxibustion group, showing significant difference as compared with the model group (P < 0.05). In contrast with the acupuncture group, jag2 decreased with significant difference (P < 0.05) in the moxibustion group.

RT-qPCR confirmed acupuncture and moxibustion regulation in the Notch signaling-related DEGs in the bone marrow cells after CTX chemotherapy.

RT-qPCR was conducted to test mRNA transcription of associated DEGs in Notch signal pathway in marrow hematopoietic cells with RT-PCR methods. The testing was performed by Genergy Bio-technology (Shanghai) Co., Ltd. The results are shown in table 2.

Results: After CTX chemotherapy, among jag1, notch2, numb1, numb2, jag1- the associated DEGs in notch signaling pathway in mice bone marrow cells, in contrast with the blank group, mRNA expression of notch2 and jag1 increased but without statistical significance (P > 0.05), and mRNA expression of numb1 and numb2 decreased, showing significant difference (P < 0.05) in the model group. Although compared with model group, the acupuncture group and moxibustion group had shown no statistical significance after treatment, mRNA expression of numb1 and numb2 raised and mRNA expression of jag1 and notch2 decreased after acupuncture and moxibustion treatment. The result of the moxibustion group is superior to that of acupuncture group, but there was no significant difference (P > 0.05).

Western Blot analysis verifying acupuncture and moxibustion impacts on the protein expression of Notch signaling-related DEGs in bone marrow cells of CTX chemotherapy-induced myelosuppressive mice.

Western blot method to test the mRNA transcription of associated DEGs in the Notch signal pathway in bone marrow hematopoietic cells. The testing was performed by Genergy Bio-technology (Shanghai) Co., Ltd. The results are shown in table 3.

Results: After injection of CTX, the content of jag1 and notch2 protein increased while the content of numb1 and numb2 protein decreased, indicating that CTX could reduce numb proteins (numb1, numb2) level in the bone marrow cells, leading to the increase of notch proteins (jag1, notch2), causing the notch signaling pathway to lose inhibition and be abnormally activated, which would result in myelosuppression. After five days of treatment, as compared with the model group, in the acupuncture group and moxibustion group, jag1 and notch2 protein content decreased, with significant difference (P < 0.05); numb2 content increased and there was significant difference in the moxibustion group (P < 0.05); numb1 protein level, though without significant difference, had showed a rising tendency (P > 0.05), and it was higher in the acupuncture group. It is indicated that acupuncture and moxibustion could increase the level of numb proteins and lower the level of notch proteins.

Discussions

Prescribed as a main mode of treatment for those patients who have lost the opportunity of surgical therapy, chemotherapy is currently a conventional method of clinical tumor treatment [15], but the therapy is associated with myelosuppression as a life-threatening toxicity in the blood system. Because of the short half-life and prompt attenuation of white blood cells, early stage of myelosuppression is manifested as leukaemia [16-18], leading to anaemia and infections, seriously affecting the living quality and prognosis of patients. Acupuncture and moxibustion treatment, with distinct curative effect and no toxicity, could relieve the myelosuppression. It has attracted increasing attention and the study of its mechanism of action has become a research hotspot.

Notch signaling pathway is an important regulator of hematopoiesis, and plays a key role in the generation and differentiation of haematopoietic stem cells (HSCs) [19-20]. Research [21] findings show that blockade of Notch signaling pathway can improve the differentiation ability of hematopoietic stem cells, whereas activation of Notch signaling can inhibit the differentiation of stem cells. Numb is a multifunctional protein found in multiple organizations and it is associated with self-renewal and differentiation functioning of progenitor cells [22]. Researches [23-24] have suggested that numb proteins would pass through and gathered at the top of the cell membrane during

<table>
<thead>
<tr>
<th>Differential gene</th>
<th>Sample size</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>jag1</td>
<td>8</td>
<td>1.8669±0.486</td>
<td>2.0719±0.4115</td>
<td>1.8853±0.6741</td>
<td>1.7297±0.5049</td>
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<tr>
<td>notch2</td>
<td>8</td>
<td>0.3055±0.1338</td>
<td>0.3390±0.0709</td>
<td>0.2566±0.0966</td>
<td>0.3249±0.1156</td>
</tr>
<tr>
<td>numb1</td>
<td>8</td>
<td>0.3261±0.1479</td>
<td>0.1732±0.0589</td>
<td>0.1825±0.1252</td>
<td>0.2826±0.1739</td>
</tr>
<tr>
<td>numb2</td>
<td>8</td>
<td>5.4033±2.2410</td>
<td>3.1032±0.5991</td>
<td>4.2528±2.2228</td>
<td>4.4376±1.5264</td>
</tr>
</tbody>
</table>

Table 2: Effect of acupuncture and moxibustion on mRNA (%) of associated DEGs in signaling pathway in CTX mice (X±s, n=8)
Note: 1) Compared with the blank group P < 0.05.

<table>
<thead>
<tr>
<th>DEGs</th>
<th>Sample size</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>jag1</td>
<td>6</td>
<td>2059.5±634.417</td>
<td>2981.0±796.30</td>
<td>207.6±546.32</td>
<td>2113.2±312.18</td>
</tr>
<tr>
<td>notch2</td>
<td>6</td>
<td>4256.9±850.04</td>
<td>5306.9±996.59</td>
<td>4294.9±572.11</td>
<td>4247.0±808.03</td>
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<tr>
<td>numb1</td>
<td>6</td>
<td>2911.8±632.6</td>
<td>2323.6±902.56</td>
<td>3065.4±849.17</td>
<td>2953.0±921.95</td>
</tr>
<tr>
<td>numb2</td>
<td>6</td>
<td>3576.1±383.26</td>
<td>2758.4±644.68</td>
<td>3318.7±364.72</td>
<td>3412.0±580.86</td>
</tr>
</tbody>
</table>

Table 3: Effect of acupuncture and moxibustion on protein level of associated DEGs in notch signaling pathway in CTX mice (X±s, n=6)
Note: 1) Compared with the blank group P < 0.05; 2) Compared with model group P < 0.05

mitosis. In case of symmetrical division along the vertical direction, they would be divided equally to produce two identical daughter cells, which will proceed to next cell cycle, to amplify and expand the progenitor cell pool; In case of asymmetrical division along the horizontal direction, two different cells shall be produced. The one without numb would continue to divide, whereas the other would maintain the state of stem cells or progenitor cells to enter the following cell cycle.

Genetic researches suggest that expression and positioning of Spdo, which is an up regulator of Notch, on Notch membrane is necessary for Notch activation. Numb could block activation of Notch by mediating Spdo endocytosis and stopping its expression on the cell membrane [25-26].

The study has showed that after the injection of CTX, mRNA expression of numb genes (numb1,numb2) in bone marrow cells dropped, giving rise to increased mRNA expression of notch genes (jag1,notch2). After acupuncture and moxibustion treatment, although acupuncture group and moxibustion group had no statistical significance compared with model group, but mRNA expression of numb1 and numb2 raised while mRNA expression of jag1 and notch2 decreased, and moxibustion group was superior to acupuncture group. Test the protein level of significant DEGs with Western Blot, numb1 protein level, though without significant difference (P > 0.05), had the trend of increasing, and the level in acupuncture group is higher than that in moxibustion group. It is indicated that acupuncture and moxibustion could lower notch protein level by increasing numb protein level.

From the analysis above, possible mechanism by which acupuncture and moxibustion relieve CTX chemotherapy induced myelosuppression may be that: CTX chemotherapy could induce decrease of numb protein (numb1,numb2) level and increase of notch2,jag1 protein level in mice bone marrow cells. Notch signal pathway loses inhibition at excessively activated state, leading to decreased proliferation of hematopoietic stem/progenitor cells in bone marrows, while differentiated cells continuing to grow. Decreased number of hematopoietic stem/progenitor cells could induce hematopoietic function ablation and myelosuppression in bone marrows. Acupuncture and moxibustion treatment to CTX mice could raise the level of Numb proteins (numb1,numb2) in bone marrow, therefore decrease the protein level of notch2 and jag1, inhibit excessive activation of Notch signaling and enhance proliferation of the hematopoietic stem/progenitor cells in bone marrow. With reduced differentiation of cells, there would be an increase of the number of hematopoietic stem/progenitor cells and enhancement of the bone marrow hematopoietic functioning, which is supposed to be the key mechanism of acupuncture and moxibustion in relieving CTX chemotherapy induced myelosuppression.

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