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Acupuncture Regulates the Notch Signaling Pathway in Bone Marrow Cells to Alleviate Cyclophosphamide-Induced Myelosuppression in S180 Tumor Bearing 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 S180 tumor bearing 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: Healthy male Kunming mice were inoculated with S180 sarcomas and further divided into blank control (group A), CTX group (group B), acupuncture (group C), and moxibustion (group D) groups. Animals in groups B, C, and D were intraperitoneally injected with single dose of 150 mg/kg CTX to generate the myelosuppressive model; while animals 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, respectively at Dazhui, Geshu, Shenshu, and Zusanli acupoints; while mice in groups A and B received no daily treatment. Animals in each group were euthanized by cervical dislocation following five days of treatment 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 up regulated while notch2 and jag1 were down regulated in bone marrow cells of acupuncture treated, tumor bearing mice.

Conclusion: Acupuncture regulated the changes of Notch signaling-related DEGs in mouse bone marrow cells. The data indicates that acupuncture treatment may alleviate the CTX chemotherapy-induced myelosuppression associated with tumor burden, suggesting that might be the key mechanism to improve bone marrow function.

Keywords: Acupuncture; Tumor bearing mice; Myelosuppression; The notch signaling pathway

Background

Chemotherapy, one of the primary treatments for malignant tumors, is commonly associated with myelosuppression-especially in the circulatory system and can thus pose a significant risk due to toxicity [1,2]. The Notch signaling pathway is closely related to many neoplastic diseases [3] and plays decisive roles in the cells and tissues (e.g., hematopoietic system, immune systems, and cancer [4]) of multicellular organisms. Normal expression and interaction of Notch molecules and their ligands maintains hematopoietic function in the body; abnormal expression may cause malignant proliferative diseases [5-8]. Our preliminary studies confirmed that acupuncture ameliorated the side effects of chemotherapy and alleviated the associated myelosuppression [9,10].

From previous studies, we simulated the myelosuppressive model in tumor bearing mice using CTX chemotherapy and further evaluated if acupuncture and moxibustion changed the protein levels and mRNA transcriptions of the Notch signaling-related differentially expressed genes (DEGs) using microarray screening, immunohistochemistry, real-time quantitative PCR (RT-qPCR), and Western Blot analysis. We aimed to identify the important signaling molecules and underlying mechanisms through which acupuncture alleviated CTX chemotherapy-induced myelosuppression in tumor bearing mice.

Materials and methods

Experimental animals

Forty, 7-week old male Kunming mice (body weight of 18 ± 2 g) were provided by Henan Laboratory Animal Center (School of Medicine, Zhengzhou University, China) and were housed in an experimental environment, with temperature between 20°C to 25°C,

humidity of approximately 60%, and unlimited access to food and water for 3 days before experimentation commenced. This study was been approved by the Ethics Committee of the School of Traditional Chinese Medicine, Henan University (Zhengzhou, China). All protocols were carried out in strict accordance with the guidelines of laboratory animal health welfare prepared by the Ministry of Science and Technology, China [11].

Model creation and treatment groups

Sarcomas were isolated from the S180 tumor bearing mice after local disinfection and submerged in normal saline for homogenization. After adjusting the cell number, a total 5 × 106 extracted cells / 0.2 ml were injected into the right armpit of each Kunming mouse.

Total white blood cells of each animal were measured from serum samples 7 days after injection, animals with body weight of 22 ± 2 g and a tumor diameter of approximately 0.8 cm were then selected for further experimentation. Thirty-two mice, with normal white cell counts, were randomly and equally divided intone of four groups (i.e., blank control: group A; model: group B; acupuncture: group C; and moxibustion: group D).

Groups were housed in distinct cages and all cages held a maximum of four animals. Animals in groups B, C, and D were intraperitoneally injected with single dose of 150 mg/kg CTX (0.2 g/bottle, Shanxi Pude Pharmaceutical Co., Ltd., China, batch number: 04120501) to generate the myelosuppressive model 4 hours after the injection (According to the pharmacokinetics of CTX, activity of CTX disappears 4 hours after administering [12]). Animals in group A were intraperitoneally injected with an equivalent volume of sterile saline (0.9% NaCl, 250 ml/bottle, Shandong Lukang Cisen Pharmaceutical Co., Ltd., China, batch number: 120412107).

Selection of acupoints

Acupoint positioning and selection were picked in accordance with the Chinese Veterinary Acupuncture and Moxibustion, and were located as follows: Daizhui (DU14) was located at the center of the back between the 7^{th} cervical vertebra and the 1st thoracic vertebra; Geshu (BL17) was located at the left and right sides of the 7^{th} thoracic vertebra; Shenshu (BL23) was located at the left and right ribs of the 2nd lumbar vertebra; and Zusanli (ST36) was located at the posterolateral side of the knees of the lower limbs approximately 5 mm below capitulum fibula.

Treatments

Animals in groups A and B were not subjected to acupuncture treatments but were situated on the bench in a similar fashion each day. Animals in group C were placed on the bench and perpendicularly pierced by acupuncture needles (specification: 0.19 cm × 10 mm with needle handle of 20 mm, Wuxi Jiajian Medical Instrument Co., Ltd., Jiangsu, China) at 3 mm depth of acupoints Daizhui (DU14), Geshu (BL17, both sides), Shenshu (BL23, both sides) and Zusanli (ST36, both sides) for 6 minutes.

Animals in group D were fixed on the bench in a similar manner before being treated with moxibustion (device speculation: 0.4 cm × 25 cm, Nanyang Bai Cao Tang Natural Moxa Products Co., Ltd., Henan, China) at a height of 2 cm on the acupoints Daizhui (DU14), Geshu (BL17, both sides), Shenshu (BL23, both sides) and Zusanli (ST36, both sides) for 3 minutes. Acupuncture and moxibustion treatments were performed once per day for five consecutive days.

Sample collection

Five days after the treatment, animals from each group were euthanized by cervical dislocation, placed on a sterilized work bench on dry ice, and their bilateral humeri and femurs were collected. After removing the attached muscle and connective tissues, humeri and femurs were rinsed with 0.9% normal saline, in to frozen storage tube, followed by storing at -80°C freezer for later use.

Statistical

SPSS16.0 statistical software (SPSS, Chicago, IL) were used for data analysis. Data presented represent the mean (χ) ± 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 4 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.

Immunohistochemistry further elucidated the Notch signaling-related DEGs in the mouse bone marrow cells in different groups:

Notch signaling-related DEGs in the mouse bone marrow cells identified by the small-throughput microarray were also analyzed using immunohistochemistry. Secondary antibody, bovine serum albumin, 3,3'-diaminobenzidine concentrated solution, neutral resin, phosphate-buffered saline, and IMS image analysis system, were purchased from Henan Huayu Biotech Co., Ltd., Zhengzhou, China. Microtome and water bath-slide drier (PPTHK-21B) were purchased from Hubei Laike Medical Instrument Co., Ltd, Hubei, China.

Detection and analysis

Through imaging and microscopy analysis, the positively stained area and optical density (OD) of the relevant regions in immunehistochemical samples were measured. After imaging, ImageJ2x software was used to calculate the average positively stained area and OD of the corresponding protein marker from the five randomly selected vision fields.

DEGs protein	Case no.	Blank control (%)	Model group (%)	Acupuncture group (%)	Moxibustion group (%)
detal1	8	739.50 ± 49.330	745.00 ± 25.037	749.00 ± 28.496	728.63 ± 23.736
notch1	8	820.38 ± 82.685	804.88 ± 73.480	807.88 ± 63.106	728.38 ± 102.096
notch2	8	788.88 ± 51.053	860.75 ± 59.2081	791.38 ± 42.6512	786.13 ± 67.9232
jag1	8	755.63 ± 39.982	821.00 ± 38.1091	743.38 ± 26.0222	773.75 ± 27.7732
jag2	8	847.13 ± 33.545	794.75 ± 37.0321	800.88 ± 42.256	816.63 ± 71.534
numb1	8	810.75 ± 53.441	741.63 ± 22.2261	785.88 ± 43.0992	790.25 ± 28.2832
numb2	8	820.00 ± 28.471	730.63 ± 17.5331	765.88 ± 25.0852	761.63 ± 41.3762
Note: [1]comparison with the blank control group (P<0.05); 2comparison with the model group (P<0.05)					

Table 1: Impacts of acupuncture on protein expression of the Notch signaling-related DEGs in CTX chemotherapy-induced myelosuppressive tumor bearing mice ($\bar{\chi} \pm s$, n=8).

Immunohistochemistical staining showed that 4 out of 7 DEGs selected based on the microarray screening were significantly different between treatment groups (Table 1). Furthermore the Notch signaling-related DEGs in the CTX chemotherapy-induced myelosuppressive tumor bearing mice were found to exhibit significantly downregulated expression of notch2, jag1, and jag2 proteins; numb1 and numb2 proteins were significantly upregulated compared with the control group (P<0.05). No significant difference of Delta1 and notch1 protein expression was found between the model and the control groups. In comparison with the control groups, notch2 and jag1 proteins in the acupuncture and the moxibustion groups were significantly downregulated; numb1 and numb2 proteins of both groups were significantly upregulated (P<0.05). No significant difference of Delta1, notch1, and jag2 protein expression was found between the groups. Therefore, jag1, notch2, num1, and numb2 proteins were examined further.

RT-qPCR confirmed acupuncture regulation in the Notch signaling-related DEGs in the mouse bone marrow cells after CTX chemotherapy:

RT-qPCR was conducted according to the manufacturer's instruction (Genergy Bio-technology, Shanghai, China) to determine the mRNA concentration of Notch signaling-related DEGs in mouse bone marrow cells. Following CTX chemotherapy, the DEGs jag1 and notch2 in the model group were slightly upregulated (no significant difference), and the DEGs numb1 (no significant difference) and numb2 (P<0.05) were downregulated, compared with the blank control group (Table 2). These results suggested that CTX chemotherapy may cause downregulation of numb1 and numb2 in bone marrow cells of tumor bearing mice. Moreover, there was a significant rise in jag1 and notch2 expression with a corresponding reduction to the Notch signaling pathway and subsequent abnormal activation of hematopoietic cells and myelosuppression. Expression of jag1 and notch2 were found to be upregulated following acupuncture or moxibustion treatment; numb1 expression was downregulated though not statistically significant when compared with the model group. Compared with the model group, expression of numb2 was significantly elevated after moxibustion (P<0.05). Although expression of numb2 in the moxibustion group was higher than in the acupuncture group, but the difference did not reach statisticale significance.

DEGs	Case no.	Blank control (%)	Model group (%)	Acupuncture group (%)	Moxibustion group (%)
jag1	8	2.8732 ± 0.6962	3.1545 ± 1.03308	2.9749 ± 0.6909	2.6503 ± 0.6233
notch2	8	0.4129 ± 0.1667	0.4378 ± 0.3066	0.4183 ± 0.2754	0.4018 ± 0.2326
numb1	8	0.2975 ± 0.1478	0.2085 ± 0.1246	0.1707 ± 0.1595	0.1707 ± 0.1595
numb2	8	5.2464 ± 2.0583	3.0733 ± 1.17231	3.3012 ± 1.06851	5.0484 ± 1.58322
Note: [1]comparison with the blank control group (P<0.05); 2comparison with the model group (P<0.05)					

Table 2: Impacts of acupuncture on the mRNA (%) of the Notch signaling-related DEGs in CTX chemotherapy-induced myelosuppressive tumor bearing mice ($\bar{\chi} \pm s$, n=8).

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

Compared with the control group, jag1 and notch2 proteins in the model group were significantly up regulated (P<0.05), and numb1 and numb2 proteins were downregulated (no significant difference, Table 3). This result suggests that CTX chemotherapy might cause the down

regulation of numb1 and numb2 proteins in the bone marrow cells of the tumor bearing mice, resulting in the elevation of jag1 and notch2 proteins and impairment of the inhibitory effect on the Notch signaling pathway, thereby causing abnormal activation of the Notch pathway in the hematopoietic cells and myelosuppression. After acupuncture or moxibustion treatment, the expression of jag1 and notch2 protein was significantly down regulated (P<0.05) compared with the model group. Protein expression of jag1 and notch2 in the moxibustion group tended to be lower than in the acupuncture group, while protein expression of numb1 and numb2 in the moxibustion group tended to be higher than in the acupuncture group, but no significant differences were found for these genes.

DEGs protein	Case no.	Blank control	Model group	Acupuncture group	Moxibustion group
jag1	6	2123.7 ± 744.09	3334.2 ± 568.03[1]	1815.2 ± 591.94[2]	1992.5 ± 317.28[2]
notch2	6	3792.0 ± 735.90	5429.9 ± 751.25[1]	4433.7 ± 508.40[2]	4516.1 ± 331.05[2]
numb1	6	3288.8 ± 1329.45	2562.4 ± 778.46	3253.4 ± 794.40	2805.3 ± 515.03
numb2	6	3968.5 ± 1023.66	2978.5 ± 932.56	3629.0 ± 1676.14	3340.1 ± 781.82
Note: [1]comparison with the blank control group (P<0.05); 2comparison with the model group (P<0.05)					

Table 3: Impacts of acupuncture on the protein quantities of the Notch signaling-related DEGs in CTX chemotherapy-induced myelosuppressive tumor bearing mice ($\bar{\chi} \pm s$, n=6).

Discussion

Myelosuppression is the most common chemotherapy-induced toxicity in cancer therapy with symptoms including varying degrees of anemia, bleeding, and infection all of which dramatically affect the patients' quality of life as well as the prognosis. Previous studies showed that the severity of myelosuppression was associated with the types, dosages, and treatment cycles of anti-cancer drugs, the renewal rate of patients' hematopoietic cells, as well as a patient's individual differences [13]. Although the current therapy for myelosuppression is fast-acting, highly effective, and broadly available, the drawbacks of high costs, multiple side effects, and unsustainable effects should not be neglected in clinical application [14]. Traditional acupuncture therapy has been linked to alleviation of myelosuppression associated with chemotherapy, and may represent a simple, inexpensive treatment.

Notch signaling plays a key role in the developmental process of hematopoietic stem cells (HSC) in vertebrates [15] in addition to controlling the differentiation of HSC and progenitor cells [16]. The Notch ligand jagged1 binds to the notch2 receptor to stimulate Notch signaling, the activation of which promotes tumor formation and malignancy [17,18]. Notch is a transmembrane receptor that controls cell fate by determining the cell development and tissue homeostasis. The adaptor protein numb acts as a notch inhibitor and an intracellular channel with known functions [19]; it is a multifunctional protein associated with self-renewal and differentiation in many organs and progenitor cells [20], and its interaction with Notch regulates cell fate [21].

Results of this study showed that numb protein (i.e., numb1 and numb2) expression was upregulated along with notch2 and jag1 protein in tumor bearing mice after CTX chemotherapy. Excessive activation of the Notch signaling pathway in the tumor bearing mice attenuated the proliferation of HSC and progenitor cells. As the number of differentiated cells continued to increase, the numbers of HSC and progenitor cells decreased. As a result, the bone marrow hematopoietic function was weakened, and the cell cycle was also affected [22], leading to myelosuppression.

In this study, microarray initially selected the DEGs, including delta1, notch1, notch2, jag1, jag2, numb1, and numb2, which related to the Notch signaling pathway in mouse bone marrow cells. Immunohistochemistry verified that notch2, jag1, numb1, and numb2 might be the most significant Notch signaling-related DEGs present in bone marrow cells. RT-qPCR revealed no significant difference in expression level of notch2, jag1, and numb1 despite notch2, and jag1 being slightly reduced and numb1 slightly elevated; numb2 expression was significantly different. Western Blot analysis verified protein expression of the Notch signaling pathway in the mouse bone marrow cells collected from the different groups. The results showed that jag1 and notch2 protein expression varied significantly in four groups. Although no significant difference was found in numb1 and numb2 protein expression between different groups by Western Blot analysis, slightly elevated numb1 and numb2 protein expression was found after acupuncture or moxibustion treatment. These findings were further verified by immunohistochemistry staining. The discrepancy between gene expression and protein expression analysis results is probably caused by the small sample size in this study, the diversity of stimulation effects in acupuncture, and the complexity of gene transcription. After CTX chemotherapy-induced myelosuppression, acupuncture or moxibustion treatment up regulated the numb protein expression (i.e., numb1 and numb2) in bone marrow of tumor bearing mice, thereby down regulating the notch2 and jag1 protein expression. These inhibited the excessive activation of the Notch signaling pathway and attenuated the CTX chemotherapy-induced myelosuppression. Therefore, we speculated that the Notch signaling pathway was an important route for the comprehensive effects of acupuncture on anti-myelosuppression, immunity enhancement and hematopoietic improvement.

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