

Research Article

Gene Expression Profiles at Moxibustioned Site (ST36): A Microarray Analysis

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As a major alternative therapy in Traditional Chinese Medicine, it has been demonstrated that moxibustion could generate a series of molecular events in blood, spleen, and brain, and so forth. However, what would happen at the moxibustioned site remained unclear. To answer this question, we performed a microarray analysis with skin tissue taken from the moxibustioned site also Zusanli acupoint (ST36) where 15-minute moxibustion stimulation was administrated. The results exhibited 145 upregulated and 72 downregulated genes which responded immediately under physiological conditions, and 255 upregulated and 243 downregulated genes under pathological conditions. Interestingly, most of the pathways and biological processes of the differentially expressed genes (DEGs) under pathological conditions get involved in immunity, while those under physiological conditions are involved in metabolism.

1. Introduction

In acupuncture research, microarray analysis has been widely employed to uncover gene expression profiles at different tissues or organs [1–19]. Based on these gene expression profiles, researchers would be able to have the possibility to find out more potentially interesting targeted genes to conduct further experiment to explain the molecular events induced by acupuncture. Moxibustion, as one of the main therapies in acupuncture clinical practice, has been demonstrated to it could be useful for pain relief [20, 21] and generated a series of molecular events in blood [22, 23], spleen [24, 25], colonic mucosa [26], brain [27], and so forth, by utilizing moxa cone or stick to stimulate acupoint or some areas (also named moxibustioned site). However, none of gene expression profiles at moxibustioned site to date has been reported. Therefore, we proposed that moxibustion could, to a considerable extent, yield a great deal of differentially

expressed genes (DEGs) at moxibustioned site, and we also anticipate to find out potential molecular targets to explain how moxibustion works at the stimulated site.

2. Material and Methods

2.1. Animals. Adult male Sprague-Dawley rats weighing 200–220 g obtained from Chengdu University of Traditional Chinese Medicine, Experimental Animal Centre, were utilized in this study. Maintained in animal room of automatically controlled day cycles (12:12 = light:dark cycle) at $24 \pm 2^\circ\text{C}$, all rats were allowed to freely take food and water *ad libitum* and randomly assigned to the various experimental groups ($n = 3$, for each group). The experimental procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and all experimental protocols were approved by

the Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine.

2.2. Experimental Design. In this study, we aimed to explore what would take place at the moxibustioned site in the view of potential molecular target under physiological and pathological conditions. Thus, we designed two different parts of microarray experiment. One is designed for uncovering the gene expression profile at physiological condition in which it consisted of 2 group: healthy control group (C) and healthy control with moxibustion stimulation group (CM). The other one is designed for revealing the gene expression profile at pathological condition in which it composed of model control group (M) and model control with moxibustion stimulation group (MM).

2.3. Intervention

2.3.1. Physiological Condition. Under physiological condition, the rats in CM group received moxibustion at the left acupoint Zusanli (ST36), at the depression below the knee from the anterior crest of the tibia [28] for 15 min. The moxibustion stimulation was manipulated with lighting moxa stick (length: 12 cm, diameter: 0.6 cm, Nanyang Hanyi Moxibustion Technology Development Co., Ltd., China) for 15 min (Figure 1). In case of skin burnt, the tip of moxa stick was kept about 2-3 cm from the skin.

2.3.2. Pathological Condition. Firstly, the pathological condition was established by injecting subcutaneously with 0.1 mL Freund's Complete Adjuvant (FCA, Sigma, USA) into the plantar surface of the left hind paw of the rat [29]. The CFA injection immediately led to local inflammation, paw swelling and pain, which became apparent within 12 hours and persisted for at least 2 weeks after injection. In this experiment, the rats in MM group received moxibustion with the same procedure as mentioned above 1 week after injection.

2.4. RNA Extraction. Two hours after one time of moxibustion stimulation was completed, rats was euthanized by CO₂ inhalation. The cutaneous tissue (0.5 cm × 0.5 cm × 0.2 cm) located at moxibustioned site were immediately removed and preserved in RNAlater (Ambion, USA) to prevent RNA degradation. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) and purified with RNA clean-up Kit (MN, Germany) following the instructions of manufacturers, respectively. Total RNA was quantitated by spectrophotometry, and the integrity was assessed by formaldehyde denatured agarose gel electrophoresis.

2.5. Microarray Analysis. The microarray analysis service provided by CapitalBio Corporation (Beijing, China) was performed as described [30, 31]. Briefly, total RNA extracted from the samples was used to produce complementary RNA using in vitro transcription technique. Then cDNA was generated by reverse transcription and used as the template to synthesize the fluorescein-labeled cDNA by Klenow fragment polymerase. Universal rat reference RNA purchased from

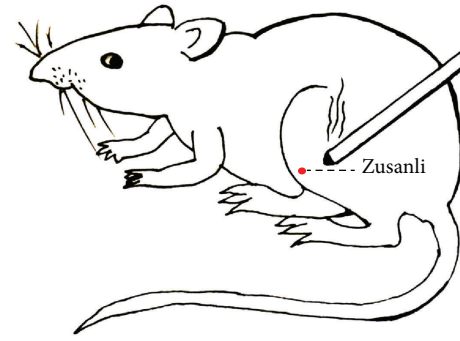


FIGURE 1: Rat received moxibustion at ST36 (Zusanli).

Stratagene was also labeled as common reference control. RNA from sample and common reference were fluorescently labeled by Cy5 or Cy3, respectively, and then were hybridized paired to 27K Rat Genome Array (CapitalBio, China). The array was comprised of 26,962 oligonucleotide probes covering 27,044 transcripts which represent about 22,012 genes. All arrays were scanned by LuxScan 10KA dual channel confocal laser scanner (CapitalBio, China). The obtained images were analyzed with LuxScan3.0 Image Analysis Software (CapitalBio, China), which employed the LOWESS normalization algorithm.

2.6. Data Analysis

2.6.1. Differentially Expressed Genes Selection. The detected signal intensities of all probes on the chip ≥ 400 were included for comparison analysis. We applied two-class unpaired algorithm in the Significant Analysis of Microarray software (SAM, Stanford) to identify significantly differentially expressed genes between CM and C groups, and MM and M groups. DEGs were determined with the threshold of false discovery rate, $FDR \leq 5\%$ and fold change ≥ 2.0 or ≤ 0.5 .

2.6.2. Pathway and Biological Processes Analysis of DEGs. We employed the online Molecule Annotation System (MAS) established by CapitalBio Corporation (<http://bioinfo.capitalbio.com/mas3/>) which integrated with KEGG and Gene Ontology (GO) database to perform pathway and GO Biological Process term enrichment analysis and calculate the statistical significance as described [32]. P value < 0.001 was considered statistically significant.

2.7. Real Time PCR Confirmation. To validate the expression patterns obtained from microarray data, we used quantitative real time polymerase chain reaction (qPCR) to detect the expression of four DEGs, Hsp90a, Mcpt8, Slpi, and Clqa, which were randomly selected from the 27K Rat Genome Array. Table 1 showed the primers designed for these genes and the housekeeping gene Gapdh. cDNA was prepared from DNase-treated total RNA using the First Strand SuperScript II Kit (Invitrogen, USA). qPCR was performed with DNA Master SYBR Green I Kit (Roche, Germany) and LightCycler machine (Roche, Germany) following the manufacturer's protocols.

TABLE 1: The primer designed for validation.

Gene	Primer (5'-3')	Temperature (°C)	Product size (bp)
Gapdh	FW: CCTTGTAAGGGCAAACCAA RV: ATGGCCTTCCGTGTTCCCTAC	59	156
Hspala	FW: GGTGAACTACAAGGGCGAGA RV: GCTGCGAGTCGTTGAAGTAG	58	152
Mcpt8	FW: CCAGGTCATCGCTGTTGTA RV: CCCAGGTTTCACCCAGTCC	62	382
Slpi	FW: ACAGACAGGGGCTCTCTTGA RV: CCTCCCAATAAGTGCCAGAA	60	216
Clqa	FW: AAGTGGGACCTTTGTCTGTCTATC RV: CCCTGCTAACACCTGGAAGAG	59	108

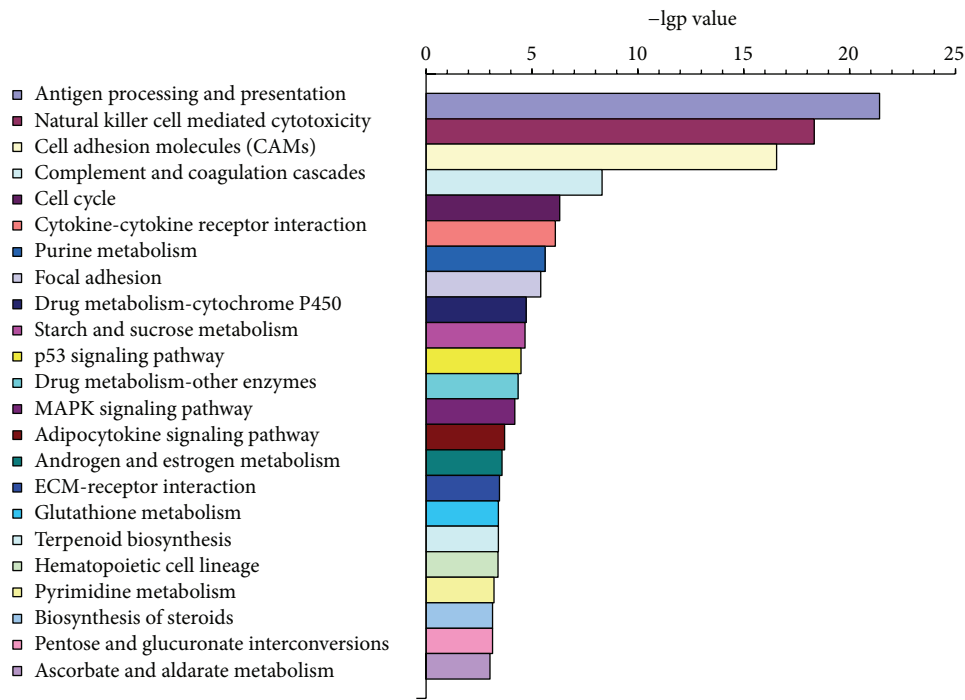
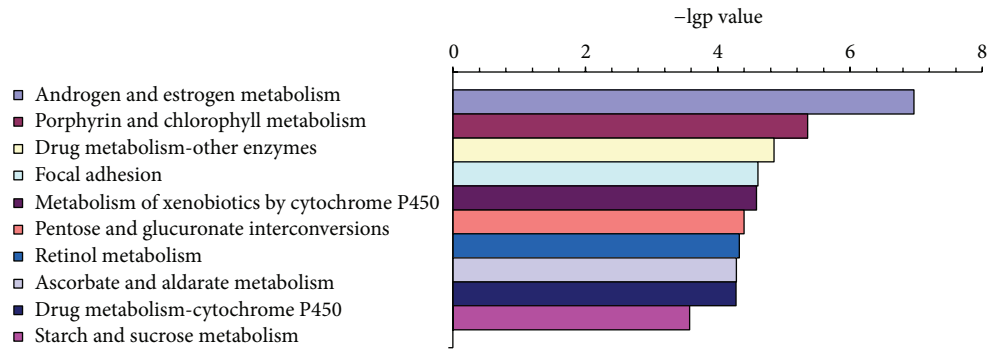
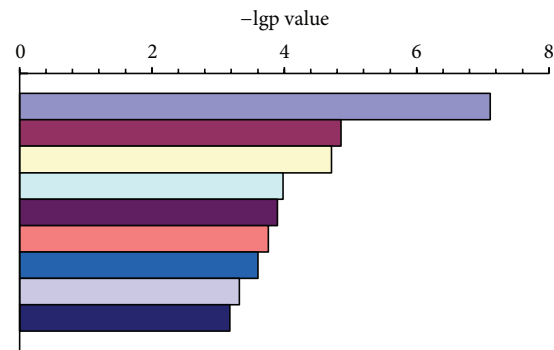


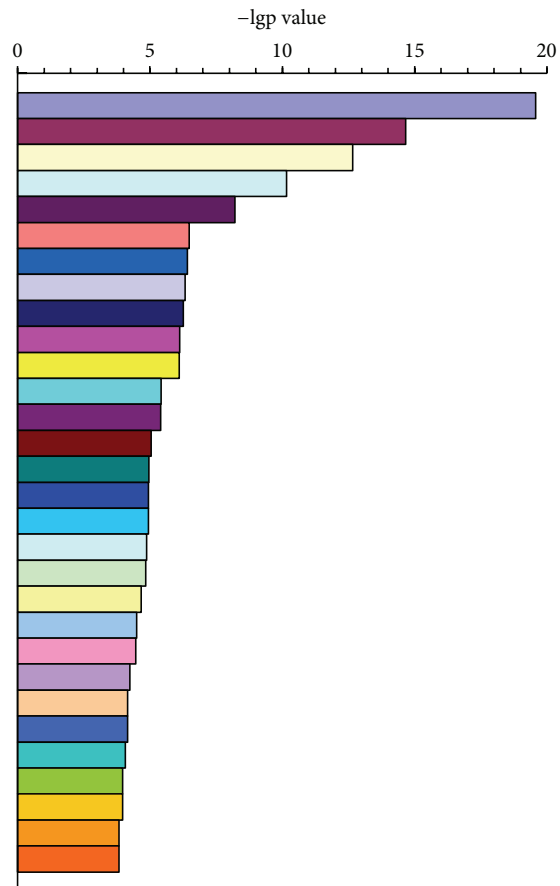
FIGURE 2: The statistically significant pathways (P value <0.001) involved in DEGs at moxibustioned site.

- Oxidation reduction
- Potassium ion transport
- Peptidyl-citrulline biosynthesis from peptidyl-arginine
- Regulation of cell adhesion
- Histidine catabolism
- Positive regulation of angiogenesis
- Response to hypoxia
- Cholesterol biosynthesis
- Catecholamine metabolism



(a) Physiological condition

- Immune response
- Antigen processing and presentation
- Antigen processing and presentation of peptide antigen via MHC-I
- Proteolysis
- Mitosis
- Protein folding
- Complement activation, classical pathway
- Response to drug
- Response to hypoxia
- Cell adhesion
- Deoxyribonucleoside diphosphate metabolism
- Cell cycle
- Cell division
- Response to heat
- Collagen catabolism
- Regulation of muscle filament sliding speed
- Extracellular matrix disassembly
- Tissue regeneration
- Regulation of cell growth
- Neutrophil chemotaxis
- Response to lipopolysaccharide
- Replication fork protection
- Cellular response to insulin stimulus
- Negative regulation of smooth muscle cell migration
- Positive regulation of osteoclast differentiation
- Response to cAMP
- Glucose homeostasis
- Calcium-mediated signaling
- Regulation of angiogenesis
- Response to hydrogen peroxide



(b) Pathological condition

FIGURE 3: The statistically significant biological processes (P value <0.001) involved in DEGs at moxibustioned site.

3. Results

3.1. DEGs at Moxibustioned Site. Different numbers of DEGs at moxibustioned site were obtained from different condition. Under physiological condition, we obtained 145 up-regulated and 72 downregulated DEGs (see Supplementary Table 1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2013/890579>). While under pathological condition, the results displayed 255 upregulated and 243 downregulated DEGs (Supplementary Table 2).

3.2. Enriched Pathways at Moxibustioned Site. Figure 2 showed us statistically significant pathways (P value <0.001)

at moxibustioned site. Under physiological condition (Figure 2(a)), it was found that 10 pathways were enriched based on all DEGs at moxibustioned Site. On the other hand, 21 enriched pathways were statistically significant under pathological condition (Figure 2(b)).

3.3. Enriched Biological Processes at Moxibustioned Site. From Figure 3, we would find out the biological processes with significantly statistical differences (P value <0.001) at moxibustioned site. Under physiological condition (Figure 3(a)), it was found out that 9 biological processes were involved. Under pathological condition (Figure 3(b)), 29 biological processes were enriched.

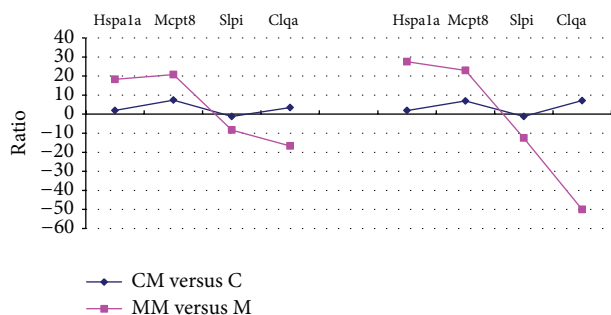


FIGURE 4: qPCR validation for 4 DEGs from microarray data.

3.4. Validation of the 4 Selected Genes. To validate the results of the microarray, we selected 4 genes, Hspa1a, Mcpt8, Slpi, and Clqa, by qPCR. The results indicated that the expression levels of confirmed genes in microarray were similar with that in qPCR (Figure 4).

4. Discussion

Here, we firstly reported the gene expression profiles at the moxibustioned site. The results imply that the moxibustioned site would also generate a setting of DEGs apart from those in other any tissue [16]. In other words, either under physiological condition or under pathological condition, we could find out a great number of DEGs responding to moxibustion stimulation at moxibustioned site. Based on these DEGs under different condition, we further seek out 22 coexpressed genes with similar expression tendency (Supplementary Table 3). To some extent, these genes (16 upregulated and 6 downregulated genes) should be most likely to be considered as potential targets for continuing studies to determine which genes would be essential or critical in the role of moxibustion at moxibustioned site.

In this study, most (8/10) of the involved pathways at moxibustioned site were related to metabolism under physiological condition (Figure 2(a)). However, the significant pathways under pathological condition induced by FCA were most associated with immunity (Figure 2(b)), such as the pathway of antigen processing and presentation and natural killer cell-mediated cytotoxicity. It is suggested that different pathway at the moxibustioned site would get involved of the in different condition even with same moxibustion stimulation.

According to the biological processes analysis, we also could find out the difference from different state. Without FCA as the pathological stimulation, the biological progresses following moxibustion administration at moxibustioned site were most composed of oxidation reduction, potassium ion transport, and so forth. However, a variety of biological processes related to immunity, such as immune response, antigen processing and presentation, and antigen processing and presentation of peptide antigen via MHC class I, and were exhibited in this study.

Taken together, it seemed to be concluded that a series of molecular events would happen at moxibustioned site. Moreover, different pathways and biological progresses at

moxibustioned site would be involved in different condition. However, this conclusion should be seriously taken for granted given the following limitations. Firstly, only one stimulation time point was used in this study. How about the time course or different time points, such as 5, 10, 15, 20, and 30 minutes, which were frequently practiced in moxibustion clinic, which needs to be answered in future study? Secondly, the pathological condition was induced by FCA injection in this study. To our knowledge, FCA injection will generate adjuvant arthritis through a series of immune actions [33, 34]. Moreover, previous studies also demonstrated that moxibustion would be useful to get better improved adjuvant arthritis [32, 35, 36]. Therefore, we cannot determine whether the DEGs at moxibustioned site under pathological condition would play important role in the moxibustion treatment of adjuvant arthritis. In this point of view, current data can only be used to explain what had happened at moxibustioned site under this condition. How about other pathological conditions? It is necessary to perform more researches to figure out the difference or similarity under different pathological conditions.

Additionally, in this study the tissue used for RNA extraction and microarray detection was taken from the skin of the moxibustioned site. So far, the skin has been also regarded as an important immune organ [37, 38] as well as a component of neuro-immuno-cutaneous system (NICS) [39–42]. In view of this aspect, we would be able to assure that the molecular event at moxibustioned site in this experiment will have the possibility to be applied to explain the initial mechanism of moxibustion activating the neuroimmune modulation which has been demonstrated in previous studies [43, 44]. This would be another potential mechanism of moxibustion apart from that it is currently considered as temperature-related and non-temperature-related work mechanisms [45].

5. Conclusions

The results suggested that a set of molecular events would have happened at moxibustioned site. Among those molecular events, different genes and different pathways and biological progresses at moxibustioned site would have got involved under different conditions.

Conflict of Interests

All authors manifest that there is no conflict of interests.

Authors' Contribution

Hai-Yan Yin and Yong Tang contributed equally to this work.

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