Gene expression microarray analysis of the spinal trigeminal nucleus in a rat model of migraine with aura☆

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Abstract

Cortical spreading depression can trigger migraine with aura and activate the trigeminal vascular system. To examine gene expression profiles in the spinal trigeminal nucleus in rats following cortical spreading depression-induced migraine with aura, a rat model was established by injection of 1 M potassium chloride, which induced cortical spreading depression. DNA microarray analysis revealed that, compared with the control group, the cortical spreading depression group showed seven upregulated genes–myosin heavy chain 1/2, myosin light chain 1, myosin light chain (phosphorylatable, fast skeletal muscle), actin alpha 1, homeobox B8, carbonic anhydrase 3 and an unknown gene. Two genes were downregulated–RGD1563441 and an unknown gene. Real-time quantitative reverse transcription-PCR and bioinformatics analysis indicated that these genes are involved in motility, cell migration, CO_2/nitric oxide homeostasis and signal transduction.

Key Words

migraine with aura; cortical spreading depression; spinal nucleus of trigeminal nerve; nervous system; potassium chloride; gene expression; cell migration; ubiquitin degradation; enzyme; regeneration; neural regeneration

Research Highlights

(1) A rat model of migraine with aura was established by injection of 1 M potassium chloride, which induced cortical spreading depression.
(2) DNA microarray analysis revealed that, compared with the control group, the cortical spreading depression group showed seven upregulated genes–myosin heavy chain 1/2, myosin light chain 1, myosin light chain (phosphorylatable, fast skeletal muscle), actin alpha 1, homeobox B8, carbonic anhydrase 3 and an unknown gene. Two genes were downregulated–RGD1563441 and an unknown gene.
(3) These nine differentially expressed genes are involved in motility, cell migration, CO_2/nitric oxide homeostasis and signal transduction.

Abbreviation

CSD, cortical spreading depression

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INTRODUCTION

Cortical spreading depression (CSD), first described in 1944[1], is characterized by a slow spreading of cellular depolarization in neurons and glial cells at a rate of 2−5 mm/min. Clinically, CSD is often accompanied by neurological diseases, such as migraine with aura, sudden convulsions, cerebral infarction and ischemic brain injury[2]. Although it is generally accepted that CSD may induce migraine with aura, the underlying mechanisms remain unclear[3-5]. The change in cerebral blood flow following CSD suggests that vasoactive molecules may play important roles in CSD-induced migraine with aura[6-8].

Several studies have examined the impact of CSD on gene expression. Following CSD, the mRNA and protein levels of nitric oxide synthase are increased in cortical astrocytes[9]. The spinal trigeminal nucleus plays an important role in migraine headaches as a primary headache nerve center. Recent studies have shown that CSD can activate the trigeminal vascular system[10]. In order to explore CSD-induced changes in gene expression in the spinal trigeminal nucleus, we first established a rat model of migraine with aura by high potassium stimulation and then conducted gene expression microarray analysis of the trigeminal vascular system. This study aimed to provide important insight into the molecular mechanism of CSD-induced migraine with aura.

RESULTS

Quantitative analysis of experimental animals

Six rats were randomly divided into two groups (n = 3): migraine group (potassium chloride stimulation group) and control group (sodium chloride stimulation group). All 6 rats were involved in the final analysis, without any dropout or loss.

Establishment of a rat model of CSD-induced migraine with aura

No CSD was recorded in the control group. In contrast, in the migraine group, CSD was observed zero to three times 1 minute after each potassium chloride injection. The total number of CSDs during six high potassium stimulations was 8.00 ± 2.65, with an amplitude of 23.47 ± 10.70 (P < 0.01, *vs.* the control group). The CSD was mainly composed of 1-minute negative waves with an amplitude of 5−40 mV, possibly surrounded by positive waves of smaller amplitude (Figure 1).
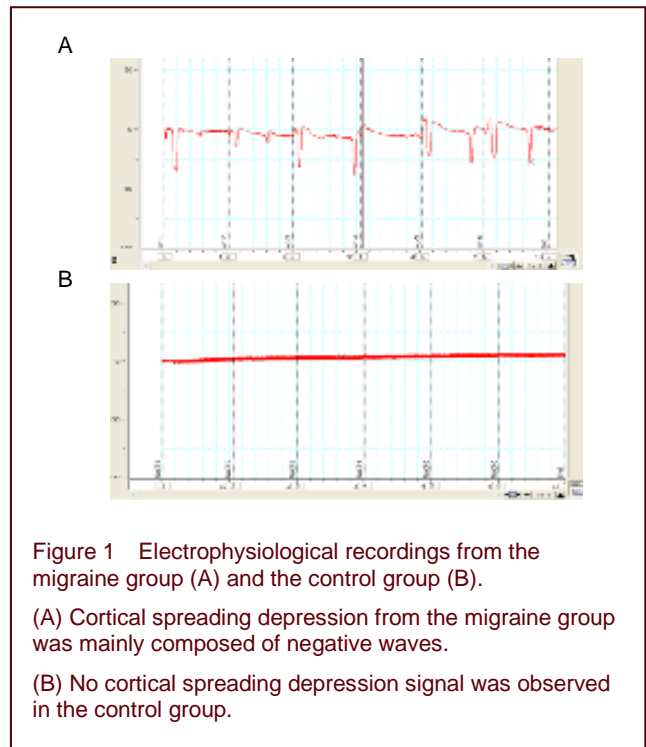

Figure 1   Electrophysiological recordings from the migraine group (A) and the control group (B).

(A) Cortical spreading depression from the migraine group was mainly composed of negative waves.

(B) No cortical spreading depression signal was observed in the control group.

Differentially expressed genes between migraine and control groups

To explore the impact of CSD on gene expression, total RNA was isolated from the spinal trigeminal nucleus, and transcriptional profiles were compared between the migraine and control groups using the Gene Chip Rat Genome 230 2.0 Array. Among a total of 31 099 probe sets, 20 718.78 ± 47.46 (66.62%) and 20 025.80 ± 379.56 (64.39%) genes were determined as "present" in the migraine group and control group, respectively, both within the normal range.

Based on Significance Analysis of Microarrays, genes with Score (d) ≥ 2, fold change ≥ 2 (upregulated in the migraine group) or fold change ≤ 0.5 (downregulated in the migraine group) were considered differentially expressed. According to these criteria, in the spinal trigeminal nucleus of the migraine group, seven genes were upregulated (P < 0.002 5) and two genes were downregulated (P > 0.997; Table 1).

Gene ontology and pathway analysis of differentially expressed genes

We analyzed these nine genes using MAS v4.0 software for gene ontology, associated pathway and functional classification. Table 2 and Figure 2 show the gene ontology analysis results, providing information on molecular function, biological process and cellular component.

Table 1　Differentially expressed genes in the spinal trigeminal nucleus between the migraine and control groups

Gene ID	Gene symbol	Chromosomal location	Fold change
1389986	–	–	2.093908↑
1370971	Myh1 /// Myh2	chr10q24	5.904218↑
1380142	Hoxb8	chr10q31	2.128121↑
1367896	Ca3	chr2q23	2.172204↑
1370033	Myl1	chr9q32	5.296848↑
1387787	Mylpf	chr1q36	5.908658↑
1369928	Acta1	chr19q12	2.947108↑
1394693	–	–	0.376251↓
1393565	RGD1563441_predicted	chr10q24	0.495874↓

↑: Up-regulated genes; ↓: down-regulated genes; based on Significance Analysis of Microarrays v4.0 software analysis.

Table 2　Gene ontology analysis of differentially expressed genes

Input symbol	Protein (↑)	Molecular function	Biological process	Cellular component
1370033	Myl1	GO: 0003774 motor activity GO: 0005509 calcium ion binding	–	GO: 0016459 myosin
1380142	Hoxb8	GO: 0043565 sequence-specific DNA binding GO:0003700 transcription factor activity	GO: 0006355 regulation of transcription, DNA-dependent GO:0007275 development	GO: 0005634 nucleus

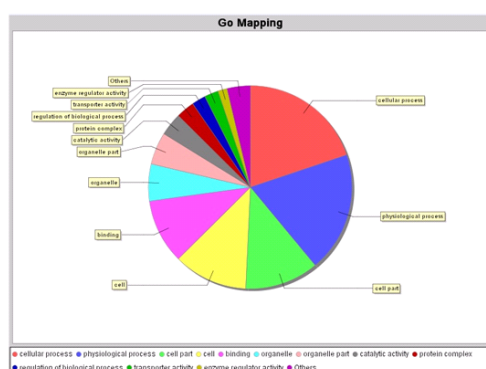Bioinformatics analysis; both genes are upregulated.

Figure 2　Ontology analysis of differentially expressed genes indicating the different functions involved.

Tables 3 and 4 summarize GenMAPP and KEGG pathway analysis, respectively.

Gene ontology and pathway analysis demonstrated that differentially expressed genes were mainly involved in cell migration, motor activity, ubiquitin degradation, enzymatic activity and transcription regulation. We observed a significant enrichment in cell migration and motor-activity related genes, especially genes associated with striated muscle and smooth muscle contraction.

Table 3　GenMAPP pathway analysis of differentially expressed genes

Pathway name	Total number (n)	Gene (↑)	Input symbol
Motor activity	3	Myl2	1387787
Motor activity	3	Mlc3	1370033
Motor activity	3	Acta1	1369928
Rn_Striated_muscle_contraction	2	Myl2	1387787
Rn_Striated_muscle_contraction	2	Acta1	1369928
Rn_Smooth_muscle_contraction	2	Myl2	1387787
Rn_Smooth_muscle_contraction	2	Acta1	1369928
Rn_G13_Signaling_Pathway	1	Mlc3	1370033
Cytoskeleton	1	Acta1	1369928
Lyase activity	1	Ca3	1367896
Ubiquitin ligase complex	1	RGD 1563441_predicted	1393565
Protein ubiquitination	1	RGD 1563441_predicted	1393565
Ubiquitin-protein ligase activity	1	RGD 1563441_predicted	1393565
Acid-amino acid ligase activity	1	RGD 1563441_predicted	1393565
Ligase activity, forming carbon-nitrogen bonds	1	RGD 1563441_predicted	1393565
Ubiquitin cycle	1	RGD 1563441_predicted	1393565

Bioinformatics analysis; genes are upregulated.

Table 4　KEGG pathway analysis of differentially expressed genes

Pathway name	Total number (n)	Gene (↑)	Input symbol
Nitrogen metabolism	1	Ca3	1367896
Regulation of actin cytoskeleton	1	Mlc3	1370033

Bioinformatics analysis; genes are upregulated.

Real-time quantitative reverse transcription-PCR results of differentiated expressed genes

We conducted real-time quantitative reverse transcription-PCR to verify the microarray analysis results for the four genes involved in cell motility-Acta1, Mylpf, Myh1 and Myl1 ($P < 0.01$). The RNA expression levels of these four genes were normalized to that of actin. Our results were consistent with the microarray results; all four genes were significantly upregulated at the RNA level

(Figure 3). The extent of upregulation based on real-time quantitative reverse transcription-PCR was much larger than that based on microarray analysis (Acta1: 71.4-fold; Mylpf: 62.5-fold; Myh1: 16.7-fold; Myl1: 41.7-fold).
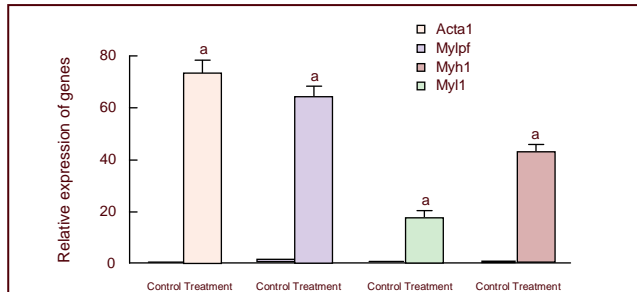

Figure 3 Real-time quantitative PCR results of differentially expressed genes.

Consistent with the microarray analysis results, real-time PCR also showed upregulation of Acta1, Mylpf, Myh1 and Myl1. [a]$P < 0.01$, *vs.* control group. Data are expressed as mean ± SD, $n = 5$ (representing 5 experiments), two-sample *t*-test.

Bioinformatics analysis of differentially expressed genes

Among the nine differentially expressed genes, two were unknown genes. Four out of the remaining seven genes were involved in motor activity and cell migration (Myh1, Myl1, Acta1 and Mylpf), and all four were upregulated. This observation indicates that in the rat model of migraine with aura, motility-related genes may be enriched to mediate signal transduction and nerve activation. We conducted bioinformatics analysis of these four motility-related genes.

Myh1, a protein of 1 912 amino acids, consists of MYSc and IQ domains that are involved in thick filament movement and calcium binding, respectively. As the heavy chain for the muscle protein myosin, Myh1 mainly participates in muscle cell differentiation and movement. Previous studies have shown that Myh1 is upregulated during inflammatory reactions[11]. To date, the potential role of Myh1 in the nervous system has not been reported.

Myl1, a protein of 189 amino acids, contains two EF-hand calcium-binding motifs. Upregulation of Myl1 has been reported in human head and neck tumors. Acta1, a protein of 377 amino acids, contains an actin domain that is mainly involved in myosin interactions. Acta1 is an important participant in cell contraction and migration. Interestingly, Acta1 interacts with Myl1[12], and similar to Myl1, Acta1 is also upregulated in human head and neck tumors. These data suggest that the four cell-motility related genes, as well as other neuromuscular molecules, may form complexes or act together to mediate signal transduction and nerve cell activation.

Mylpf, a protein of 169 amino acids, contains two EF-hand motifs as well and mainly participates in calcium binding.

We also performed bioinformatics analysis on the other three known genes.

Homeobox B8, a protein of 282 amino acids, is a homeodomain transcription factor of the Homeobox family. Studies on transgenic mice have shown that mutations in Homeobox B8 can lead to abnormalities in neuronal development[13-15].

Carbonic anhydrase 3, a protein of 260 amino acids, contains a carbonic anhydrase domain. Carbonic anhydrase 3 belongs to a class of carbonic anhydrases that catalyze the reversible hydration of carbon dioxide.

RGD1563441, a protein of 190 amino acids of unknown function, is mainly expressed in the brain and prostate. Its N-terminus contains a RING finger domain, a domain that is characteristic of E3 ubiquitin ligases[16-19]. Future studies are required to clarify its functions.

DISCUSSION

Migraine is a common chronic disabling neurological disorder with recurrent pulse-like pain, usually lasting from several hours to 3 days[20-22]. Migraine is often accompanied by nausea, vomiting, photophobia and phonophobia[23]. Approximately 15% of patients experience aura, a transient neurological disturbance before or during headache onset. Both human and rat studies have suggested that CSD is involved in the pathogenesis of migraine with aura[1, 24]. CSD activates the metalloproteinase system, including enzymes that increase vascular permeability, which in turn activates the trigeminal vascular system[25]. Based on these previous findings, we induced CSD in rats to establish an animal model of migraine with aura.

CSD can be induced by focal stimulation of the gray matter of the cerebral cortex, hippocampus or cerebellum. The underlying mechanism may be related to a rapid change in ion concentration, which is caused by secretion of K^+ and H^+ as well as uptake of Na^+, Ca^{2+} and Cl^-[26]. Common inducers in CSD models include high K^+ levels, excitatory amino acids, acupuncture and electrical stimulation. In this study, we induced CSD by microinjecting high concentration K^+ solutions into the cortex. After high concentration K^+ solution injection, electrophysiological recording demonstrated significant inhibition and reversal of electric signals, showing successful establishment of an animal model of migraine with aura. The molecular mechanism of migraine with aura is not clear. It is commonly believed that CSD may affect gene

expression in the brain, leading to physiological and pathological phenotypes. A few target genes have been identified, including nitric oxide synthase, c-jun and c-Fos. However, most studies have focused on a single pathway; thus, a genome-wide picture is not available. Gene expression chips provide an invaluable tool for effectively examining and comparing gene expression profiles from different subjects (healthy and diseased), during different developmental stages, or under different stimulation conditions. Gene Chip Rat Genome 230 2.0, the gene chip used in this study, covers 31 099 transcripts and 28 000 rat genes, providing a comprehensive perspective on the mechanism of CSD-induced migraine with aura. One previous study reported a genome-wide microarray of whole brain tissue in a rat CSD model[27]. Our study casts further insight into this topic by using medulla oblongata and upper cervical tissues, rather than the whole brain tissue, as the study material. The spinal trigeminal nucleus plays an important role in migraine headache as the primary headache center and can be activated by CSD[10]. In order to reveal the molecular mechanism, we used microarray gene chip (Affymetrix Rat Genome 230 2.0 Arrays) analysis of CSD in the spinal trigeminal nucleus of rats.

In our study, 2 018 genes were identified by the chips, accounting for 66.62% of the whole genome, indicating that the coverage is effective. We used a 2-fold change as the threshold for both up- and down-regulation and identified nine affected genes, with seven being upregulated and two being downregulated. The number of affected genes was fewer than expected. There are three possible reasons. First, 1 M potassium chloride was used to induce CSD in our animal model of migraine with aura. This dose is high enough to induce CSD, but it may not be sufficient to affect gene transcription on a large scale, particularly as there is a time lag between stimulation and the initiation/shut-down of gene transcription. However, the relatively low dose used in our study has the advantage of decreasing the false-positive rate of the genes identified, and this effect was confirmed by our real time-PCR analysis. In addition, the medulla oblongata and upper cervical tissues used in our study contain a variety of cell types, including neurons, glial cells and vascular endothelial cells. Different types of cells may respond to CSD differently, which may dilute the difference in gene transcription patterns. This is a common problem for microarray analyses and may not be addressed easily in the near future. Further studies, in which specific tissues or cell types are isolated for gene transcription analysis, will provide more valuable information. Furthermore, our stimulation lasted for 1 hour, which is shorter than the duration used in previous stu-

dies (2 hours or more) and may also account for the fact that relatively fewer genes were identified. Our rationale was that in patients with migraine with aura, the typical duration of aura ranges from 5 to 20 minutes or longer, although usually shorter than 1 hour[28]. Therefore, when using CSD as a trigger of migraine with aura, a 1-hour stimulation period better mimics the clinical situation. Four genes found to be upregulated by microarray were consistent with reverse transcription-PCR results. Four of the six upregulated genes of known function are highly expressed in striated and smooth muscle cells and are involved in cell migration and mobility. Many migraine patients exhibit nausea and vomiting, and these symptoms are usually worsened after physical activities. Our findings suggest that nerve muscle cells may play a role in CSD-induced migraine with aura.

One upregulated gene, carbonic anhydrase 3, is involved in nitrogen metabolism[29]. Nitric oxide, a neurotransmitter, participates in signal transduction in the central and peripheral nervous systems[30]. *In vivo*, the synthesis of nitric oxide from L-arginine is catalyzed by the enzyme nitric oxide synthase. One particular form of nitric oxide synthase, neuronal nitric oxide synthase, is especially important in generating nitric oxide for the transmission of pain signals in the central nervous system. Recent clinical studies have shown that when nitroglycerin was applied either sublingually or intravenously, both migraine patients and non-migraine patients showed short-term dose-dependent headache[31]. In addition, migraine patients experienced migraine-like episodes, one to several hours after nitroglycerin intake. Since the half-life of nitroglycerin is very short (only 3–4 minutes), such a delayed migraine-like headache is likely to result from the influence of nitric oxide, the metabolite of nitroglycerin, rather than a direct nitroglycerin-induced vasodilator effect. Our findings suggest that CSD may be involved in the pathogenesis of migraine with aura by impacting nitrogen metabolism.

For the new gene RGD1563441, bioinformatics analysis showed that it contains a RING finger domain, a characteristic domain for RING-type E3 ubiquitin ligases. Interestingly, many pathological processes in the nervous system are associated with disturbances in the ubiquitin system, with the most prominent example being Parkinson's disease. A key gene involved in the pathogenesis of Parkinson's disease is parkin, a RING-type E3 ubiquitin ligase[32-33]. The new gene RGD1563441 is similar to parkin, which also belongs to the RING E3 family with brain-specific expression patterns. Until now, no ubiquitin-related molecules have been associated with CSD or migraine. We plan to further determine the function of RGD1563441 to reveal its potential role in

CSD-induced migraine with aura.

Future studies are required to refine the genome-wide picture for CSD-induced migraine with aura. Real time-PCR and *in situ* hybridization are both useful tools for validating data from microarray analysis. Our study provides important insight into the pathogenesis of CSD-induced migraine with aura and paves the way for future studies on migraine.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
This study was performed in Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences, China from 2010 to 2011.

Materials
Six healthy male Sprague-Dawley rats, weighing 200–250 g, were provided by the Experimental Animal Center of the PLA Military Academy of Medical Sciences (Beijing, China; license No. SCXK-(army)2007-004). Rats were housed under a 12-hour light/dark cycles with *ad libitum* access to food and water. Experimental protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[34].

Methods
Establishment of a rat model of migraine with aura
Rats were anesthetized with 20% urethane (1.2–1.5 g/kg, intraperitoneal injection) and fixed on a stereotaxic instrument. Their body temperature was maintained at 36.5 ± 0.5°C. To expose the skull, a central incision was made, and the superficial fascia was excised. On the left hemisphere, two holes (diameter 1.8 mm) were made on the skull using an electric drill, one at 3 mm anterior and 2 mm medial-lateral from the bregma and the other at 6 mm posterior and 5 mm medial-lateral from the bregma. The dura mater was maintained intact during this process. At the 3-mm-anterior hole, a glass electrode (tip diameter 1 µm; filled with 0.5 M sodium acetate solution) was inserted 1 mm below the surface of the cortex to record the CSD signal. A silver electrode was placed on the neck skin. At the 6 mm-posterior hole, 5 µL of 1 M potassium chloride (migraine group) or 5 µL of 1 M sodium chloride (control group) was injected using a microsyringe, 1 mm under the cerebral cortex. A total of six

injections were conducted, with a 10-minute interval between the injections.

Changes in the electrical activity were recorded using a PowerLab system (AD Instruments, Bella Vista, Australia). CSD was defined as a rapid emergence of a potential change with amplitude greater than 5 mV[35]. Recorded electrophysiological parameters included the total number of CSDs during six stimulations and the CSD amplitude. All models were successfully established in the CSD group.

Preparation and scanning of DNA microarray samples
Gene expression profiling in the migraine and control groups was performed using the GeneChip Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA, USA). The microarray sample was prepared following the manufacturer's protocols. Briefly, after high potassium stimulation, rats were quickly decapitated, and the medulla oblongata and upper cervical region (to T_2) were removed and stored in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The quantity of total RNA was determined by spectrophotometric analysis, and the quality was examined by electrophoresis. RNA was then reverse transcribed using a T7-Oligo(dT) primer and synthesized into double-stranded cDNA. Using the purified double-stranded cDNAs as templates, biotinylated cRNAs were synthesized, fragmented into 35–200 nucleotide pieces, and hybridized for 16 hours to an Affymetrix array at 45°C with constant rotation (60 r/min). The array was stained and washed in the Affymetrix Fluidics Station and then scanned using a GeneChip Scanner 3000.

Analysis on DNA microarray data
The microarray scanning data were analyzed using Affymetrix GeneChip Operating Software version 1.4. The output from the GeneChip Operating Software was further analyzed using dChip 2006 software (www.dchip.org)[36-37]. For each probe set, a detection P-value of < 0.04 indicated "present"; 0.04–0.06: "marginal"; and > 0.06: "absent". When comparing gene expression in the presence or absence of CSD, a change in P-value (migraine group *vs.* control group) of < 0.002 5 reflected a significant increase in gene expression in the migraine group; 0.002 5–0.003 0: a marginal increase; 0.003 0–0.997: no change; 0.997–0.997 5: a marginal decrease; and > 0.997 5: a significant decrease. To evaluate the relative abundance of gene transcripts, the signal log ratio was calculated using the one-step Tukey's biweight method.

Differentially expressed genes were identified using Significance Analysis of Microarrays software by cluster analysis. These genes were further examined using the Molecule Annotation System v4.0 software (Capital Bio Corporation, Beijing, China) for gene ontology analysis, pathway analysis and functional classification.

Real-time quantitative reverse transcription-PCR

Total RNA was isolated using Trizol reagent. SYBR Green real-time PCR was conducted following standard protocols. Specifically, the PCR reaction system (20 μL) contained 1 μL of template cDNA, 1.6 μL of $MgCl_2$, 2 μL of DNA Master SYBR Green I Mix, as well as forward and reverse primers. The PCR primers were synthesized by CapitalBio Corporation and the sequence was as indicated in Table 5. The real-time PCR conditions were as follows: a cycle of 10 minutes at 95°C; and 40 cycles of 15 seconds at 95°C, 15 seconds at 50–60°C (depending on the primers), 20 seconds at 72°C, and 3 seconds at 76°C. Fluorescence signals were measured at the end of annealing in each cycle with the critical point for measurement declined during PCR amplification. A melting curve analysis was performed using a pattern of 95°C for 15 seconds, 60°C for 20 seconds, and 95°C for 15 seconds.

Table 5　PCR primers used for amplification of genes

Gene	Primer sequence (5' –3')
Actin	F: GTA CCC AGG CAT TGC TGA CA R: CTC CTG CTT GCT GAT CCA CAT C
Actin (alpha 1)	F: TTG AAC CCC AAA GCT AAC CG R: GTC CCC AGA ATC AAA CAC GA
Myosin light chain, phosphorylatable, fast skeletal muscle	F: GAA CAG GGA TGG CAT TAT TGA C R: GAC CTT GAA GGC TCC AGT GAT
Myosin light chain 1	F: GGG CAC CAA TCC CAC AAA T R: ACA CGC AGA CCC TCA ACG A
Myosin heavy chain 1	F: GGA GAA GTT GAA AAC GAA CAG AA R: CTT GTA TGC TTT GAC CTT TGA TTG

F: Forward; R: reverse.

Bioinformatics analysis

Genetic information sources included http://www.ncbi.nlm.nih.gov and http://www.ensembl.org. Protein domain analysis was performed at http://smart.embl-heidelberg.de/. Chip information was obtained from the NCBI Gene Expression Omnibus database website.

Statistical analysis

Data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. For microarray data analysis, two sample *t*-test was employed when two groups were compared. A *P* value < 0.05 was considered to be statistically significant.

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(Edited by Zhang CL, Jia DL/Yang Y/Song LP)