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Original Article

Altered Expression Profiling of Spinal Genes Modulated by Compound 48/80 in A Mouse Itch Model

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ABSTRACT

Background: The molecular mechanisms underlying itch are constantly being studied to create new opportunities to prevent or alleviate itch. The aim of our study was to determine the spinal gene expression changes induced by compound 48/80 in a mouse itch model.

Methods: Mice were divided into saline group (control group, n=12) and compound 48/80 group (48/80 group, n=12). 100 μl saline or compound 48/80 was microinjected intradermally in the nape of the neck. After injection, pruritic behavior was immediately measured every 5 minutes. 30 minutes after injection, tissue was prepared to carry out mRNA profiling microarray and reverse transcription polymerase chain reaction (RT-qPCR) analyses.

Results: The total numbers of scratching bout after compound 48/80 injection (100 μg/100 μl) were significantly increased in 48/80 group (190 ± 11.33) as compared with control group (6.83 ± 1.17). We screened the dorsal part of the cervical spinal cord of the mouse itch model for differentially expressed genes. Out of 45037 studied transcripts, the abundance levels of 15 transcripts were altered following compound 48/80 injection. 9 and 6 genes were up- and down-regulated in 48/80 group, respectively. We validated the reliability of the microarray results by RT-qPCR, and found 6 up-regulated mRNA, including Sgk1, Bag4, Fos, Ehd2, Edn3 and Wdfy, were significantly increased, whereas 3 down-regulated mRNA, including Corin, 4921511E18Rik and 4930423020Rik, were significantly decreased.

Conclusions: These findings indicate that the alterations of spinal gene expression are involved in acute itch, and provide a translational bridge for spinal drugs targeting their signaling pathway to prevent or alleviate itch.

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Obstinate itching is intolerable and difficult to manage (1-3). Some pruritus represent several serious skin diseases such as atopic dermatitis, as well as significant clinical problems resulting from liver diseases and renal diseases. Despite its clinical importance, the underlying mechanisms of the itch are not fully understood (4). It is well-known that the itch varies among individuals, such that they can be classified as histamine-dependent and histamine-independent itch (5, 6). Previous studies have demonstrated that the dorsal horn of the spinal cord, with long projections to primary afferents located in the dorsal root ganglia, is critical for mediating the itch sensation (7-11).

In the present study, we explored the spinal gene profiling which may be involved in itch by using the Affymetrix Mouse Genome 430 2.0 microarray platform. The aim of our study was to determine the spinal gene expression

changes induced by compound 48/80 in a mouse itch model. The genes associated with compound 48/80- induced itch should provide insight into the etiology of itch and identify novel therapeutic targets.

MATERIALS AND METHODS

Animals and Ethics Statement

C57BL/6 mice (8- 10 weeks old) were tested, each weighing between 20 g and 25 g, and housed in groups of four under a 12 h light/dark cycle, and maintained in a barrier facility at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. All experiments were performed on C57BL/6J mice. The experimental procedures were executed in accordance with the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (No.TJ-A20150803).

Experimental Design

Mice were divided into saline group (control group, n=12) and compound 48/80 group (48/80 group, n=12). 100 μl saline or compound 48/80 was microinjected intradermally in the nape of the neck. After injection, pruritic behavior was immediately measured every 5 minutes. 30 minutes after injection, tissue was prepared. All procedures were conducted in an isolated quiet room to reduce variance.

Behavioral Assay

Mice were adapted to the testing situation for at least 15 minutes. They were briefly anesthetized with isoflurane (2% in 100% oxygen), and received an intradermal microinjection of 100 μl compound 48/80 (100 μg/100 μl) or saline in the nape of the neck (via a 0.3 ml insulin syringe with a 31 gauge needle). As previously described (12- 14), immediately after the injection, the mouse was placed into the arena and videotaped from above for 30 minutes. Videotapes were reviewed by the observers of the pruritic behaviors. All investigators were blind to the treatment of the mice, and the number of scratch bouts was recorded at 5 minutes intervals. All pruritic behavior was evaluated as previously described with minor modifications (13, 15, 16).

Tissue Preparation

After pruritic behavior assay, the animals were immediately decapitated by cervical dislocation. Following decapitation, the dorsal part of cervical 5-8 segments in spinal cord were dissected and removed. The tissue was flash frozen in liquid nitrogen. Total RNA was isolated using Trizol® reagent (Invitrogen, Carlsbad CA). RNA samples were performed by Ambion mirVana miRNA Isolation Kit for purity and concentration.

mRNA Profiling Microarray Analyses

High quality samples containing 2 μg of total RNA were used on microarray chips. Gene profiling of C5- 8 spinal cord from control group and 48/80 group were carried out using standard Affymetrix protocols and hybridized to Affymetrix Mouse Genome 430 2.0 Array as described previously (17).

Real-Time Quantitative PCR

Total RNA from 12 male mice was provided for quantitative RT-PCR. The methods for quantitative PCR (Q-PCR) were essentially as described previously (18, 19). The constitutively expressed β- actin gene was used as the internal control to test with RT- qPCR for all analysis. The forward (F) and reverse (R) primers (Table 1) were designed using the primer express software (version 2.0- PE Applied Biosystems). Experiments were evaluated in triplicate.

Statistics and Data Analysis

Data were expressed as means ± standard error of the mean (SEM). To test for differences between the two groups, Mann-Whitney test were applied for each gene. In case the ANOVA was significant ($P<0.05$), a paired Student's t-test was applied as a post- hoc test to test for differences between control group and 48/80 group.

RESULTS

Grouping and Evaluation

We examined the scratching behaviors in male C57BL/6J mice after intradermal injection of pruritic agent compound 48/80 into the nape of

Table 1. Primer Sequences for RT-qPCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
Sgk1	TGCCACCCTGGATCTATAACTG	GGCCTCAAAGTCTGACTCCC
Bag4	CCAAACACCTACCGTTCACCT	TGTGGTCGTCCAGTCCCTC
Aqp7	AAGGGCTTTCGTGCATCAGTA	ACTCCTATCCAGAAAACCGTCAA
Sept2	GGGTGGTGACAGTGACAGCG	CCTTCCTTCCACAGGGCTAA
Edn3	GCTTGCGTTGTACTTGTATGGG	GGTGGGCTTTATCTGTCCTTGA
Wdfy1	ATCAAGACCTATCCAGCCCACC	AAGCCCACGACGAGAAGAAG
Corin	AAAAGCGACCGAGATAAGAGTG	AAGCGCAGCAAGTTAGCAGT
Tubb2a-ps2	TGCATTTTGATGCCTTAGAAGT	CGCATGGTGCCTGGTTAG
Reln	CTTTGATGGCTTGCTGGTGA	GGTTGGTTGTAGGCAGGTGA
Fos	GCCCCTTCTCAACGACCC	CATCCCCAAGGAATTGCTGT
Ehd2	CGCAAGCTCAACGACCTAGT	TGAAGTCATGTGCCATCAACAG
4930423O20Rik	CTCAGCACCGACTCTTACACG	TGCTCTTGCTTCTTGCTCCTA
4921511E18	CGTCCCTGACCCCTACTCC	AAACTAGCAAGTGGCCCGTTA
β-actin	CACGATGGAGGGGCCGGACTCATC	TAAAGACCTCTATGCCAACACAGT

the neck. As compared with control group, mice in 48/80 group exhibited a dramatic increase in scratching behaviors (Figure 1; Mann-Whitney test; $P<0.01$).

Identification of Differentially Expressed Genes

Analysis of the microarray data showed that a total of 15 genes were differentially expressed between control group and 48/80 group, in which 9 genes were up-regulated and 6 genes were down-regulated (Table 2). The maximal and minimal fold change was 5.1625 and 2.0098, respectively.

Real-Time RT-PCR

To validate the reliability of the microarray results, we analyzed these differentially expressed (DE) mRNAs, including 8 up-regulated mRNA and 4 down-regulated mRNA, by RT-qPCR (Figure 2). The dorsal part of spinal cord (C5-C8) tissues were collected from control group and 48/80 group. Six up-regulated mRNA, including Sgk1, Bag4, Fos, Ehd2, Edn3 and Wdfy (Figure 2A-F) were significantly increased, and three down-regulated mRNA, including Corin, 4921511E18Rik and 4930423020Rik (Figure 2G-Ii) were significantly decreased. RT-qPCR results of three mRNA, including Aqp7, 2-sep and Reln (Figure 2J-L) were not consistent with data from microarray.

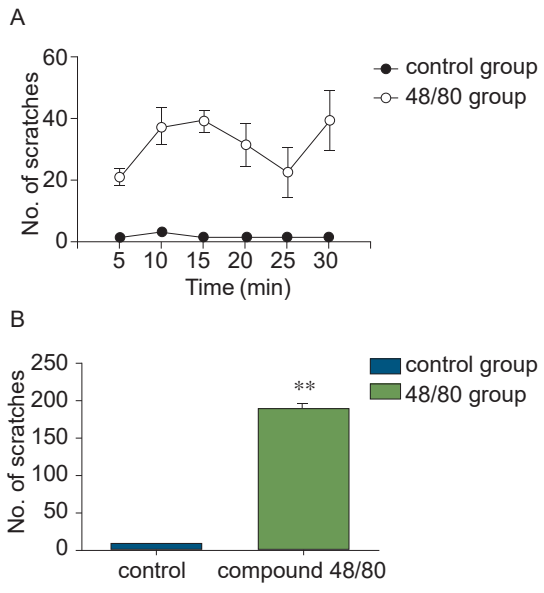

Figure 1. Scratching Behaviors in Control Group and 48/80 Group.
A. The time course shows bouts of scratching at 5-min intervals; B. The total numbers of scratching bout at the 0-30 min observation period after compound 48/80 injection (100 μg/100 μl) were significantly increased in 48/80 group (190±11.33) as compared with control group (6.83±1.17). Mann-Whitney test. $^{**}P<0.01$.

DISCUSSION

Intractable pruritus remains a major public health problem, and little can be done to reverse the initial vicious itch-scratch cycles, which significantly reduce quality of life for those afflicted. To develop intervention strategies to limit the itch-scratch-itch circuit damage, more studies need to be done to decipher the biological mechanisms of intense itch and urge to scratch.

Table 2. List of Genes Which were Differentially Expressed in Spinal Cord of the Mouse Itch Model.

Genebank ID	Fold Change	Gene Symbol	Gene Description	Molecular Function
NM_001161845	3.3041 ↑	Sgk1	Serum/glucocorticoid regulated kinase 1	The AGC family of serine/threonine kinases
NM_026121	2.204 ↑	Bag4	BCL2-associated athanogene 4	The antiapoptotic BAG family proteins
NM_007473	5.1625 ↑	Aqp7	Aquaporin 7	The glycerol channels
NM_010234	2.1018 ↑	Fos	FBJ osteosarcoma oncogene	A functional marker of neuronal activation
NM_001159717	2.0098 ↑	2-Sep	Septin 2	Septin cytoskeletal proteins
NM_010891	2.1288 ↑	Ehd2	EH-domain containing 2	The C-terminal EH domain containing a protein family
NM_007903	2.2467 ↑	Edn3	Endothelin 3	Endothelin-3/endothelin receptor B signaling
NM_001111279	2.162 ↑	Wdfy1	WD repeat and FYVE domain containing 1	
NM_001122756	0.4324 ↓	Corin	Corin	
NR_003964	0.4194 ↓	Tubb2a-ps2	"tubulin, beta 2a, pseudogene 2"	
Mm.158476.1	0.3279 ↓	4921511E18Rik	RIKEN cDNA 4921511E18 gene	
Mm.195687.1	0.4466 ↓	4930423O20Rik	RIKEN cDNA 4930423O20 gene	
NM_011261	0.4385 ↓	Reln	Reelin	

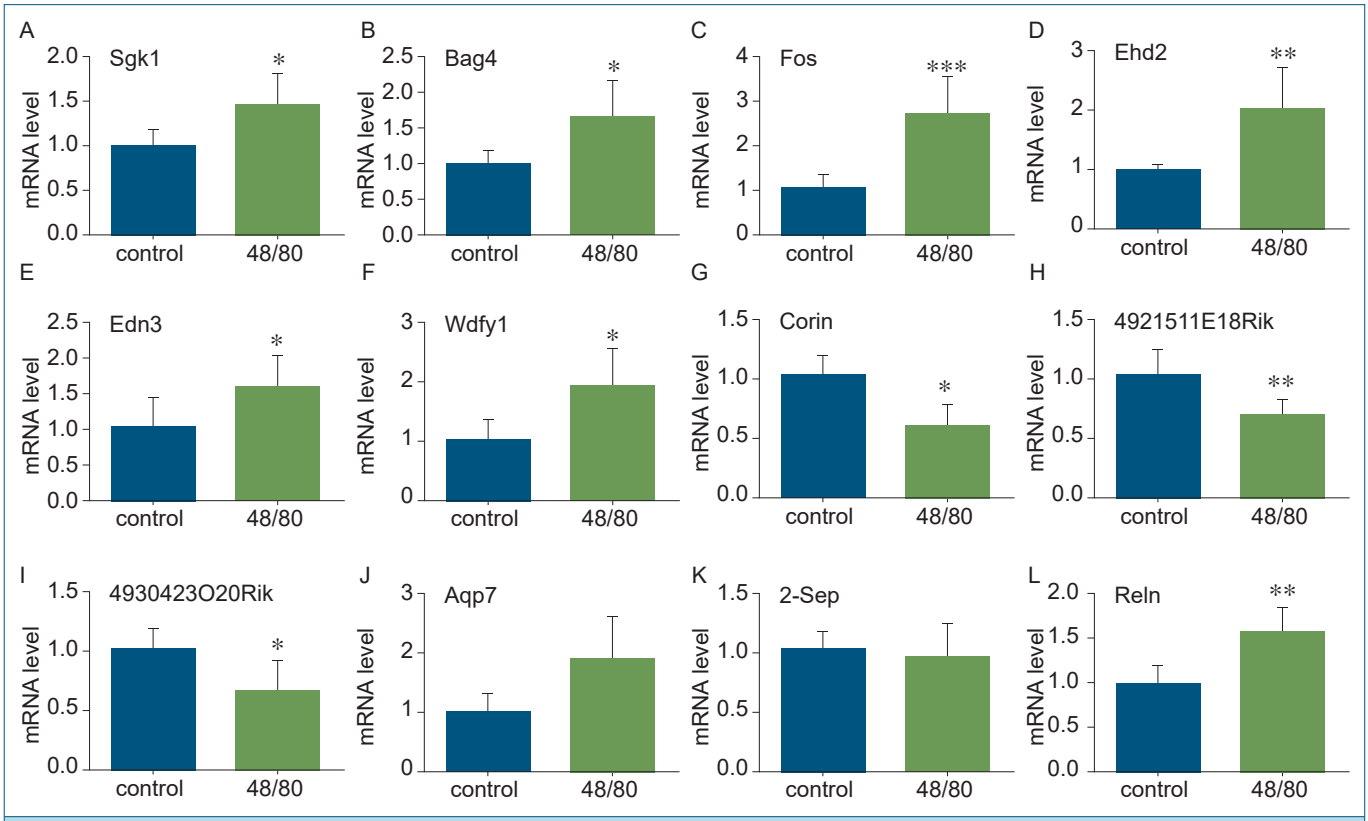

Figure 2. Real-time Quantitative PCR (qPCR) Validation of 12 Deregulated mRNAs in the Spinal Cord from Compound 48/80-Evoked Pruritus Mice.
The expressions of mRNA Sgk1 (A), Bag4 (B), Fos (C), Ehd2 (D), Edn3 (E), Wdfy1 (F) and Reln (L) were significantly up-regulated underlying compound 48/80-evoked pruritus. The expression of mRNA Corin (G), 4921511E18Rik (H) and 4930423O20Rik (I) was significantly down-regulated. The expression of mRNA Aqp7 and 2-Sep (Figure 1J, k) had no statistically different between control group and itch group. Mann-Whitney test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P< 0.001$.

Our experiments demonstrated that compound 48/80 produced itch sensation and induced the increased scratching behaviors in male C57BL/6J mice.

Our results indicated that 9 and 6 genes from 45037 studied transcripts were up- and down-regulated in itch model, respectively. We validated the reliability of the microarray results by RT-

qPCR, and found 6 up-regulated mRNA, including Serum/glucocorticoid regulated kinase 1 (Sgk1), Bcl- 2- associated athanogene 4 (Bag4), Fos, EH-domain containing 2 (Ehd2), endothelin 3(Edn3), and WD repeat and FYVE domain containing 1(Wdfy1), were significantly increased, whereas 3 down-regulated mRNA, including Corin, 4921511E18Rik and 4930423020Rik, were significantly decreased. The study of Won et al showed protein kinase Sgk1 enhanced MEK/ERK complex formation through the phosphorylation of ERK2 (20). Lee et al reported that Sgk1 was a target of the MAPK/ERK signaling pathway (21). Zhang et al reported that compound 48/80-induced itch sensation is required for activation of ERK signaling in the spinal cord, and found that ERK activation, as revealed by anti- phosphorylated ERK1/2 immunostaining, is observed in the spinal dorsal horn of mice treated with intradermal injections of histamine and compound 48/80 but not chloroquine or SLIGRL- NH2, indicating that ERK activation only occurs in histamine-dependent acute itch (22). These data were consistent with our results describing Sgk1was markedly up- regulated in mouse responding to compound 48/80.

Taken together, our data provide a global view of the genetic differences in the spinal gene expression changes induced by compound 48/80 in a mouse itch model. These findings are expected to contribute to our understanding of the differential changes of these genes in the spinal cord of patients with intractable pruritus to elucidate the etiology of itch and develop novel therapeutic targets.

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