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Molecular Changes Involving MEK3–p38 MAPK Activation in Chronic Masticatory Myalgia

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Abstract

The exact mechanism underlying chronic masticatory myalgia (CMM), a conspicuous symptom in temporomandibular disorders, remains unclear. This investigation compared gene expression profiles between CMM patients and healthy subjects. Peripheral blood leukocytes were collected in 8 cases and 8 controls and subjected to whole genome microarray analyses. Data were analyzed with Gene Ontology and interactive pathways analyses. According to Gene Ontology analysis, categories such as ion transport, response to stimuli, and metabolic process were upregulated. The pathway analysis suggested overexpression of the mitogen-activated protein kinase (MAPK) pathway in CMM patients and to a higher degree in a pathway network. Overexpression of representative members of the MAPK pathway—including MAPK kinase 3 (*MEK3*), calcium voltage-gated channel auxiliary subunit gamma 2 (*CACNG2*), and growth arrest and DNA damage–inducible gamma (*GADD45G*)—was validated with real-time polymerase chain reaction. The upregulation of *MEK3* was negatively correlated with the age of the CMM group. In the next step, the authors focused on *MEK3*, the gene that exhibited the greatest degree of differential expression, and its downstream target protein p38 MAPK. The results revealed upregulation of MEK3, as well as phosphorylated MEK3 and phosphorylated p38 MAPK, in CMM patients. These results provide a "fingerprint" for mechanistic studies of CMM in the future and highlight the importance of MEK3–p38 MAPK activation in CMM.

Keywords: temporomandibular disorders, microarray analysis, chronic pain, gene ontology, leukocytes, *CACNG2*

Introduction

Pain of the masticatory muscles, such as chronic masticatory myalgia (CMM), is one of the myogenous disorders under the temporomandibular disorder (TMD) family (Kimos et al. 2007). These chronic conditions are often affected by jaw movement, function, or parafunction and commonly cause disability (Schiffman et al. 2014). CMM represents a significant health care issue that frequently results in patients seeking either primary care (dental or medical) or referral to specialist services. However, the etiology and underlying pathogenetic mechanisms of CMM are still not fully understood.

Over the past decades, pain research has been increasingly conducted at a level that integrates system biology with cellular and molecular investigation (Gu et al. 2005). Genetic risk factors play an important role in the etiology of chronic pain conditions, putatively by modulating the underlying processes, such as inflammation, nociceptive sensitivity, psychological well-being, and autonomic responses (Nielsen et al. 2008; Smith et al. 2011). Advances in molecular biology—such as microarray techniques that enable researchers to detect the relative expression levels of thousands of genes in a single experiment—have become the most common approach for studying gene functions, pathway dissection, and drug evaluation (DeRisi et al. 1996; Golub et al. 1999; Momen-Heravi et al. 2014). Recently, this technology has been increasingly used to examine genetic risk factors and therapeutic targets for pain-related diseases that often overlap TMDs (Aaron and Buchwald 2003), such as chronic fatigue syndrome (Kerr et al. 2008) and fibromyalgia (Smith et al. 2012). However, its potential value in CMM has not been explored.

Blood cells travel throughout the body and respond to internal and external signals. Previous studies identified differential gene expression in peripheral blood leukocytes (PBLs) in

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A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

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patients with complex regional pain syndrome (Kaufmann et al. 2007). A study using peripheral blood samples identified an association of pathway activity with the immune response and homeostasis in fibromyalgia patients (Lukkahatai et al. 2015). This finding may contribute to a better understanding of the pathophysiology of pain progression in these patients.

The present study was designed to identify novel target genes involved in CMM by including samples from 8 CMM patients and 8 healthy controls in the initial screening experiment. The expression of multiple genes was examined at an mRNA level through microarray analysis. The results revealed changes in multiple genes and pathways. Candidate genes and proteins in the mitogen-activated protein kinase (MAPK) pathway were verified with real-time polymerase chain reaction (PCR) and Western blotting methods. Our discovery of the upregulation of MAPK kinase 3 (MEK3) at the gene and protein levels in CMM patients provides insights into the molecular mechanisms underlying the pathogenesis of this disorder.

Materials and Methods

Research Subjects and Diagnostic Classification

The current study was conducted in accordance with the Declaration of Helsinki. CMM patients were recruited from the Center for TMD and Orofacial Pain of Peking University School and Hospital of Stomatology. The diagnosis of CMM was established with the "Diagnostic Criteria for Temporomandibular Disorders" protocol published by the International RDC/TMD Consortium Network and Orofacial Pain Special Interest Group (Schiffman et al. 2014). Specifically, the criteria for the diagnosis included the following: 1) confirmation of pain location in the temporalis or masseter muscle; 2) report of familiar pain with maximum unassisted or assisted opening movement or palpation of the temporalis or masseter muscle; 3) pain corresponding to an intensity score ≥3 on a visual analog scale of 0 to 10 (Wewers and Lowe 1990) and duration of at least 3 mo; 4) chronic temporalis or masseter pain not attributable to recent acute trauma or previous infection; and 5) chronic pain in the temporalis or masseter muscle not attributable to active inflammation. Patients with ≥1 of the following conditions were excluded: inflammatory, rheumatic, or otherwise painful disease (e.g., fibromyalgia or low back pain); dental, sinus, or other infection; medication with steroids, narcotic agents, or antidepressants; primary psychiatric disease; previous temporomandibular joint surgery; epilepsy, cardiac, renal, or hepatic disorder; clinical evidence of dental or periodontal disease. Pregnant or nursing women were not included. All subjects were 18 to 70 y of age.

Age- and sex-matched healthy individuals (18 to 70 y of age) were recruited from Peking University students and staff by distribution of flyers. Exclusion criteria for healthy participants included a medical/psychological problem, prescribed medication, pregnancy or nursing, and a recent history of pain at any location.

A total of 43 consecutive CMM patients were included in this study. PBLs were collected from all subjects for RNA and protein profiling. All individuals provided written informed consent for blood collection and use. The study protocol was approved by the Ethics Committee of the Peking University Health Science Center (PKUSSIRB-2013012).

Blood Sampling and Processing for Gene Analysis

A total of 4 to 10 mL of peripheral blood was collected from each participant in an ethylenediaminetetraacetic acid (EDTA) vacutainer. PBLs were separated and lysed in TRIzol (Life Technologies). RNA quality and purity were analyzed by spectrophotometry with the NanoDrop ND-1000 (NanoDrop Technologies) and denaturing gel electrophoresis immediately before microarray processing.

Whole-genome microarray analysis of PBLs from a subset of 16 subjects (8 CMM cases and 8 controls) was conducted. All microarrays were processed in the same laboratory by 1 person following standard operating protocols to minimize nonbiological technical bias. cDNA was generated by use of One-Cycle Target Labeling and Control Reagents (Affymetrix), and cRNA was created with a GeneChip IVT Labeling Kit (Affymetrix). Biotin-labeled, fragmented (≤200 nucleotides) cRNA was hybridized for 16 h at 45 °C to Affymetrix GeneChip Human Gene 2.0 ST arrays. The arrays were washed, stained, and read by the GeneChip Scanner 3000 (Affymetrix). Fluorescence excitation was at 570 nm, and all the data were collected on a confocal scanner at 3-μm resolution. GeneChip Operating Software 1.4 (Affymetrix) was applied to analyze the data. We applied the random variance model *t* test to filter differentially expressed genes for the control and CMM groups because this *t* test can raise the degrees of freedom when the sample size is small (Wright and Simon 2003). After the significant and false discovery rate analyses, we selected the differentially expressed genes according to the *P* value threshold; $P < 0.05$ was considered statistically significant (Clarke et al. 2008).

Gene Ontology Category and Pathway Analyses

Differentially expressed genes were organized into hierarchical categories through Gene Ontology (GO; http://www.geneontology.org/). Significant genes were then analyzed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). Details regarding these GO and pathway analyses are described in the Appendix.

Real-Time PCR Assays

Total RNA was isolated from the PBLs of all 43 patients and age- and sex-matched controls via the same protocol as described for the microarray assay. cDNA was synthesized from 1 μg of total RNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR analyses and data collection were performed on the ABI 7500 PCR system (Applied Biosystems) through the primer pairs listed in Appendix Table 1. The reactions were performed with an ABI StepOne real-time PCR system with the following steps: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min in 96-well plates in a volume of 20 μL containing SYBR Green

PCR Master Mix (Applied Biosystems). Differentiation of mRNA expression was analyzed by the comparative Ct method (Schmittgen and Livak 2008) with the 18S rRNA housekeeping gene as reference. Lower deltaCt values indicated higher gene expression (Uceyler et al. 2015).

Protein Extraction and Western Blotting Analyses

Proteins were extracted from the PBLs of all participants, with radio immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Beyotime Biotechnology). The cell lysates were centrifuged at 15,000 *g* at 4 °C for 20 min. The supernatant was collected and measured for protein concentration with a bicinchoninic acid protein assay kit (Beyotime Biotechnology). For each sample, 50 μg of protein was loaded per lane onto a 5% to 8% polyacrylamide gel and then transferred to a polyvinylidine difluoride membrane (Millipore). After blocking with 5% nonfat milk and 0.1% Tween-20 in Tris-buffered saline at room temperature for 1 h, the polyvinylidine difluoride membranes were incubated at 4 °C overnight with 1 of the following primary anti-rabbit antibodies: anti-MEK3 (1:1,000; Abcam), anti-p-MEK3 (1:1,000; Abcam), anti-p38 (1:1,000; Cell Signaling Technology), anti-p-p38 (1:1,000; Cell Signaling), and anti-β-actin (1:2,000; ComWin Biotechnology). This was followed by incubation for 2 h with a horseradish peroxidase–conjugated secondary antibody (1:10,000; Proteintech Group). The membranes were developed in an enhanced chemiluminescence solution (Beyotime Biotechnology) for 2 min and examined with a luminescent image analyzer (Fusion FX; Vilber Lourmat). The intensity of immunopositive bands was quantified with ImageJ 1.38 software (National Institutes of Health). Protein levels were standardized to β-actin as an internal control.

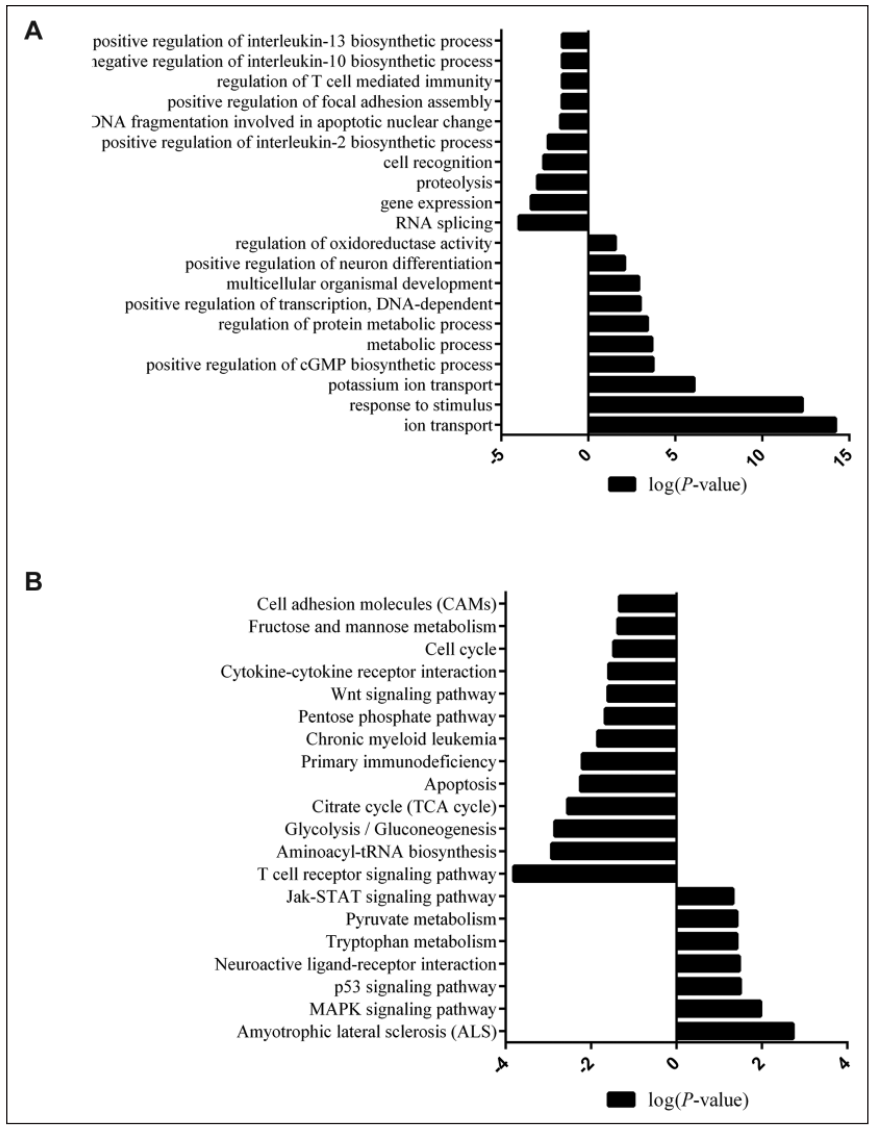

Figure 1. Gene Ontology (GO) categories and pathways in chronic masticatory myalgia patients versus controls. (**A**) The 10 most significantly upregulated and downregulated GO categories identified from microarray data of chronic masticatory myalgia patients versus controls. The significant GO category is based on the biological process for upregulated or downregulated genes. (**B**) Significant altered pathways in a pathway network are shown. Log *P* is the base-10 logarithm of the *P* value.

Statistical Analyses

Continuous variables with normal distribution and equal variances were analyzed with Student's *t* test and are expressed as means ± SEM. Continuous variables not following normal distribution or with unequal variances were analyzed with the nonparametric Mann-Whitney *U* test and are presented as the median and interquartile range. Spearman's correlation was used to assess correlations between variables. All statistical analyses were performed with SPSS 20.0 software (IBM). $P < 0.05$ was considered statistically significant.

Results

Bioinformatics Analysis of Microarray Data

Expression analysis with the mRNA microarrays was performed on PBLs from 8 CMM patients and their corresponding controls as an initial screening tool and to guide subsequent gene expression studies. Of the 28,869 probes tested, 934 were differentially expressed, with 432 upregulated and 502 downregulated in the PBLs of CMM patients versus controls. Hierarchical cluster analysis is shown in Appendix Figure 1. Analysis by the GO database showed that the GO categories upregulated significantly in CMM patients involved ion transport, response to stimuli, and metabolic process (Fig. 1A). The MAPK signaling, p53 signaling, and Jak-STAT signaling pathways played especially important roles in CMM patients, as

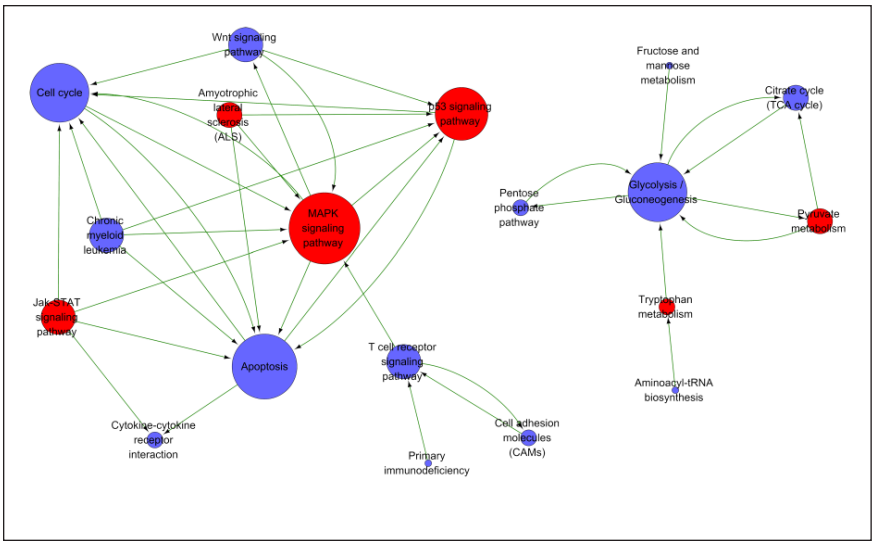

Figure 2. Pathway enrichment and interaction network analyses of chronic masticatory myalgia patients and controls. Interactions between the significantly changed pathways are connected in a pathway network. Each pathway was measured by counting the upstream and downstream pathways, which are illustrated as indegree, outdegree, or degree. A higher degree indicates that this pathway regulates or was regulated by other pathways, suggesting a more important role in the pathway network. Pathways colored in red are upregulated; pathways colored in blue are downregulated; and node size means the node degrees. Relations are indicated by arrows. MAPK, mitogen-activated protein kinase; TCA, tricarboxylic acid.

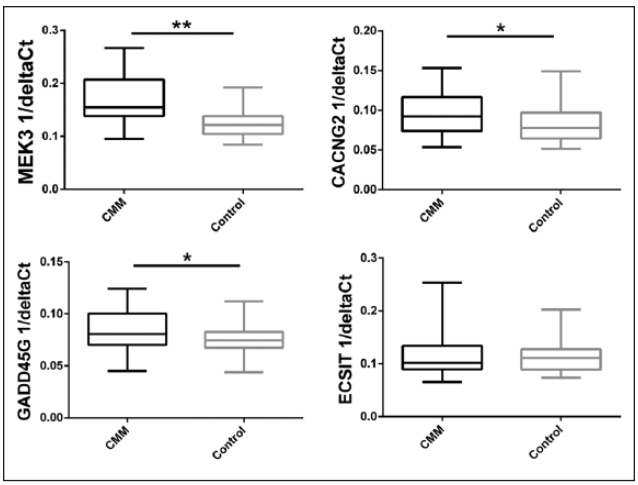

Figure 3. Confirmation of *MEK3, CACNG2, GADD45G*, and *ECSIT* gene expression in chronic masticatory myalgia (CMM) patients and controls by real-time polymerase chain reaction. Lower deltaCt values indicate higher gene expression. The results are illustrated as 1/deltaCt in boxplots to show the reciprocal value for intuitive data presentation. *$P < 0.05$. **$P < 0.01$. $n = 43$ for both CMM and control groups.

analyzed by the KEGG pathways database (Fig. 1B). Significantly changed pathways were connected in a pathway network (Fig. 2).

Confirmation of Differential Gene Expression by Real-Time PCR

Results from the microarray experiments were validated with the entire sample (86 subjects). Subject characteristics and visual analog scale scores are shown in Appendix Table 2. We focused on the MAPK pathway and selected 4 genes (*MEK3, CACNG2, GADD45G*, and *ECSIT*) on the basis of their fold change in microarray, molecular function, and biologic process for validation (Appendix Table 3). Three of the 4 genes (excluding *ECSIT*) upregulated in the microarray analysis were confirmed to have significantly higher mRNA expression by real-time PCR (Mann-Whitney U test, $P < 0.05$; Fig. 3). *MEK3* had the highest fold change (Appendix Table 3). The upregulation of *MEK3* was negatively correlated with age in the CMM group (Spearman's correlation, $r = -0.359$, $P = 0.018$). There was no correlation between pain intensity (or duration) and relative gene expression for any of the genes.

Protein Analysis of MEK3, p-MEK3, p38 MAPK, and p-p38 MAPK

Among the 3 overexpressed genes, we assessed the expression of the MEK3 protein and its downstream target protein p38 MAPK. The protein levels of MEK3, p-MEK3, and p-p38 MAPK were significantly higher in CMM patients than in the controls. Changes in the expression of p38 MAPK did not reach statistical significance (Fig. 4). No correlation was found between protein expression and other evaluated parameters.

Discussion

Preliminary microarray results in the current study showed differences in a variety of interesting genes, functions, and pathways between CMM patients and healthy subjects. Availability of the initial screening bioinformatic data set should invite further analyses to identify typical nuclear genes associated with CMM. The GO and KEGG pathway databases were used for biological process enrichment analyses. Differentially expressed genes were enriched in several GO categories, including ion transport, response to stimuli, and metabolic process. The upregulation of these GO terms suggest that these biological processes are involved in the pathogenesis of CMM. At the pathway level, we identified a significant upregulation of the MAPK pathway, including overexpression of the *MEK3, CACNG2*, and *GADD45G* genes. Upregulation of MEK3 in CMM patients was detected at both the mRNA and protein levels. Also, phosphorylated MEK3 was elevated in CMM patients relative to that of controls, adding further support to an increase in activation of MEK3. These results are generally consistent with a previous study indicating that MEK3 is necessary for the development of chronic pain following spinal sensitization in mice (Sorkin et al. 2009). However, in our

study, we have demonstrated for the first time in humans that MEK3 is involved in the pathogenesis of CMM.

Although peripheral blood is a complex tissue, previous studies revealed relatively restricted inter- and within-individual variation in gene expression when examined by microarray analysis, and they suggested that this variance was remarkably less than that observed in disease states (Whitney et al. 2003). In this study, we controlled for several confounding influences on peripheral blood gene expression, including age, sex, and medication use.

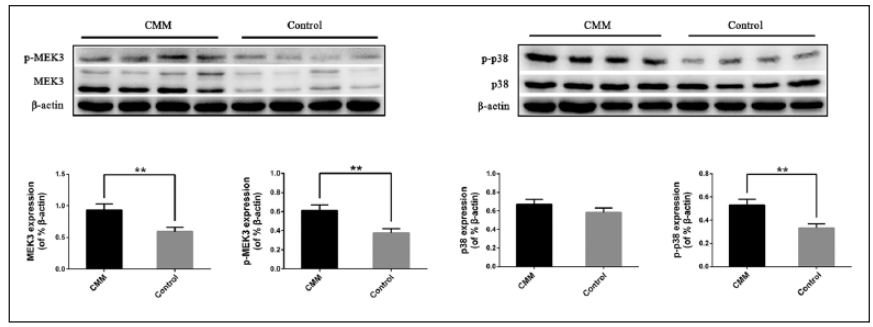

Figure 4. Increased activation of the MEK3–p38 MAPK signaling pathway in the chronic masticatory myalgia (CMM) patient group. The phosphorylation and protein levels of MEK3 and p38 MAPK were determined by Western blotting, with β-actin serving as a loading control (upper panels). Relative protein levels of MEK3, p-MEK3, p38 MAPK, and p-p38 MAPK were normalized to β-actin. Data are expressed as mean ± SEM. $^{**}P < 0.01$. $n = 43$ for both the CMM and control groups (lower panels). MAPK, mitogen-activated protein kinase; MEK3, MAPK kinase 3.

Pathway-based methods are powerful tools for investigating various biological phenomena at the system or functional level (Ishii et al. 2008). The results from our study revealed overrepresentation of the MAPK pathway and a higher degree of interaction in the pathway network in CMM patients. This pathway is activated under a variety of persistent pain conditions and leads to the induction of pain hypersensitivity through transcriptional or nontranscriptional regulation (Ishii et al. 2008; Ji et al. 2009). Moreover, MAPKs play an important role in the progression to chronic pain (Patil and Kirkwood 2007). A study revealed that rats with spinal cord injuries required activation of p38 MAPK to progress to chronic central neuropathic pain (Crown et al. 2006). Other authors also suggested that inhibition of p38 MAPK reversed mechanical allodynia and thermal hyperalgesia in chronic neuropathic pain (Anand et al. 2011; Obata et al. 2004). In addition, our previous research on rats demonstrated that MAPKs play significant roles in facial pain induced by occlusal interference (Cao et al. 2013).

On the basis of these previous works, we validated the expressions of 4 candidate MAPK genes by real-time PCR. The results showed that *MEK3* was overexpressed with a higher fold change in CMM patients. Although most patients were women of child-bearing age, consistent with previous reports (Greenspan et al. 2007), a negative correlation was identified between *MEK3* upregulation and age among CMM patients. However, we failed to find a similar correlation at the protein level, despite the fact that the MEK3 protein was upregulated significantly in the CMM group—possibly because the quantitative relationship between mRNA levels and those of the encoded protein is complex and not well understood, even after investigation with the best available techniques (de Sousa Abreu et al. 2009; Ostlund and Sonnhammer 2012). In addition, we failed to find a correlation between gene expression and pain intensity, possibly due to the relatively narrow range of pain intensities among patients: 3 to 4 (on a 10-point visual analog scale) in most patients with CMM. MEK3 is activated by mitogenic or stress-inducing stimuli and participates in the MAPK signaling cascade. Once MEK3 is activated, it specifically phosphorylates and activates p38 MAPK, which in turn participates in a variety of cellular processes, such as proliferation, differentiation, and transcription regulation (Derijard et al. 1995), and induces a variety of intracellular responses associated with neuropathic and other types of chronic pain (Raman et al. 2007; Lin et al. 2014).

Several studies have defined the mechanisms by which p38 MAPK is activated in neural tissues in animal models of pain (Crown et al. 2006; Zhuang et al. 2007; Ji et al. 2009). Another study suggested that MEK3 was necessary for the development of chronic pain and phosphorylation of spinal p38 MAPK (Sorkin et al. 2009). To further analyze the mechanisms underlying CMM, we assessed the protein levels of p38 MAPK in CMM and control groups. In our work, we failed to find a significant difference in the protein levels of p38 MAPK between the 2 groups. However, the level of p-p38 MAPK was significantly higher in the CMM patients, indicating that p38 MAPK could participate in the pathogenesis of CMM through its activation to the phosphorylated form instead of upregulation of total p38 MAPK protein levels.

P38 MAPK is a crucial contributor for pain relief in several chronic pain conditions, and inhibitors of this kinase have been tested for their effectiveness against pain in animal models (Ji and Suter 2007). However, such inhibitors may produce cardiovascular and psychiatric side effects or liver toxicity (Ji and Suter 2007; Pradal et al. 2015). The results of the current study show that MEK3 is crucial in mediating the activation of p38 MAPK in CMM patients. Therefore, blocking its activation or expression may be a promising alternative for pharmacologic treatment of CMM.

In addition to changes in *MEK3* gene regulation, our study demonstrated upregulation of *CACNG2* and *GADD45G* at the mRNA level in CMM patients. However, we failed to observe any changes in the expression of *ECSIT* by real-time PCR, possibly due to the limited number of samples.

CACNG2 encodes the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor regulatory protein, which may also serve as a calcium channel subunit involved in the trafficking of glutamatergic AMPA receptors and the modulation of their ion channel functions. The GO analysis suggested that the upregulation of ion transport contributed to the pathogenesis of CMM. Although first described as an epileptic factor, *CACNG2*

was reported as a novel neuropathic pain gene affecting pain sensitivity both in mice and in humans (Nissenbaum 2012). The current results are consistent with prior reports that *CACNG2* is one of the pain-related genes in chronic fatigue syndrome, which shares many common features as well as mechanistic underpinnings with TMDs (Aaron et al. 2000; Fang et al. 2006).

GADD45G is a gene whose transcript levels are increased following stress-related growth arrest or treatment with DNA-damaging agents. This gene participates in the ontogeny of the central or peripheral nervous systems and regulates anatomic development and neuronal differentiation (Huang et al. 2010). Although little is known about the role of *GADD45G* in CMM, the upregulated expression of this gene indicates that *GADD45G* may contribute to pain hypersensitivity in CMM.

Taken together, the results of the current study provide a preliminary description of the genetic fingerprints of CMM. To our knowledge, this is the first study that systematically investigates gene expression in PBLs of CMM patients. Major findings include increased expression of *MEK3, CACNG2*, and *GADD45G* mRNA in CMM patients. Elevated protein levels of MEK3, p-MEK3, and p-p38 MAPK indicated that activation of the MEK3–p38 MAPK pathway is important in CMM.

Limitations of our study include the case-control design and the relatively small sample population. Furthermore, the study design (inclusion of only patients with CMM and healthy controls) prevents us from specifically attributing the identified changes to CMM. Therefore, large-scale longitudinal cohort studies including other chronic pain conditions are required to confirm our findings.

Author Contributions

H. Meng contributed to the conception, design, data analysis, and acquisition and drafted the manuscript; Y. Gao and Y.F. Kang contributed to data analysis and drafted the manuscript; Y.P. Zhao, G.J. Yang, and Y. Wang contributed to acquisition and drafted the manuscript; Y. Cao and Y.H. Gan contributed to interpretation and drafted the manuscript; Q.F. Xie contributed to conception, design, analysis, interpretation, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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