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Voltage-gated sodium channels gene expression in Burning Mouth Syndrome: a case-control study

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Abstract: Burning mouth syndrome (BMS) is a condition characterized by painful symptoms of the oral mucosa, despite the absence of any clinical signs. Its etiology is unknown, and there is still no effective treatment to date. Current evidence has shown neuropathic impairment in BMS patients. Neuropathic pain can be related to the dysfunction of voltage-gated sodium channels, considering that these receptors regulate the induction of action potentials in nociceptive neurons. This study evaluated the gene expression of voltage-gated sodium channels $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ in these patients. The gene expressions of these channels were assessed by real time RT-PCR analysis of fresh-frozen tongue biopsies in a case-control study composed of 12 patients with BMS, and 5 healthy control patients, proportionally matched by sex and age, and analyzed using the 2^(-Delta Delta CT) method. There was no statistically significant difference between the analyzed groups, despite the increase in $Na_v1.7$ (fold-change = 3.13, p = 0.52) and decrease in $Na_v1.9$ (fold-change = 0.45, p = 0.36) gene expression in the BMS group. The $Na_v1.8$ gene was not expressed in any of the samples analyzed. Although the gene expression in the voltage-gated sodium channels in BMS under study seems to be comparable with that of the normal oral mucosa, the functionality of these channels in BMS has not yet been identified, thus suggesting that further research is needed to better understand these voltage-gated sodium channels.

Keywords: Burning Mouth Syndrome; Voltage-Gated Sodium Channels; Gene Expression.

Introduction

Burning mouth syndrome (BMS) is characterized by a spontaneous sensation of burning pain, or dysesthesia, in clinically normal oral mucosa, lasting for a period of more than two hours a day, for at least 3 months. This condition is more prevalent in women, usually at menopausal or postmenopausal age after the 6th decade of life.[1] The diagnosis should rule out local or systemic diseases that can cause similar symptoms,[2] such as hyposalivation, oral candidiasis, diabetes and others conditions. BMS management is still a challenge to oral medicine professionals. Conventional therapy is mainly based on antidepressants, and photobiomodulation is gaining visibility as an alternative therapy.[3]

Regarding the etiology of BMS, psychological and psychiatric factors trigger the onset, and provoke the progression of the burning symptoms.[4] However, some findings have included impaired functions of the peripheral and/or central nervous system in BMS etiology, such as decreased small-fiber density of sensitive epithelial and subpapillary nerves,[5,6] heat allodynia and tactile hypoesthesia,[7] alteration in the blink reflex,[8] and central impairment of the dopaminergic system.[9] These essential nosological characteristics highlight the possible neuropathic origin of BMS.

An investigation into the biomarkers of significant involvement in BMS onset or progression could better clarify its etiological bases, and promote the development of more specific and effective therapeutic strategies. Some research has been conducted on salivary proteins associated with inflammation and stress.[10-12] Biomarkers related to the pain mechanism have been studied only sparingly in BMS. Changes in gene and/or protein expression that may be relevant to BMS include those in the calcitonin gene-related peptide that modulates nociceptive transmission in the trigeminovascular system;[13] in opiorphin, a natural inhibitor of enkephalin-inactivating ectopeptidases that suppress the pain sensation derived from both chemical and acute mechanical stimuli;[14,15] and in the nerve growth factor, which plays an important role in the nociceptive function.[16]

Voltage-gated sodium channels are involved in the induction of the action potentials of nociceptive neurons from the sodium influx that occurs when these channels are opened. Impaired function and mutations in voltage-gated sodium channels, especially $Na_v1.7$ and $Na_v1.9$, are known to affect pain sensitivity in syndromes and neuropathic conditions, and are potential therapeutic targets for pain control in these diseases.[17-24] So far, only two studies have evaluated the expression of voltage-gated sodium channels ($Na_v1.7$ and $Na_v1.8$) in BMS .[6,18] Thus, the aim of this study was to evaluate the expression of the genes encoding voltage-gated sodium channels $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$, by quantitative reverse transcription–polymerase chain reaction (qRT–PCR), in patients diagnosed with BMS.

Methodology

Patients and samples

This case-control study included a convenience sample of 12 BMS patients (12 women, age range: 48–71 years, mean age: 61.4 ± 7.7) diagnosed according to the definition established by the International Association for the Study of Pain (IASP),[1] and to the following additional inclusion criteria: daily burning symptom in the tongue, for a period lasting at least 4 months, and no history of treatment for this symptom at least 30 days prior to sample collection. Five patients (4 women and 1 men, age range: 48–80 years, mean age: 63.4 ± 12.2), proportionally matched by sex and age, with benign tongue lesion (fibrous hyperplasia) composed the control group.

The patients excluded from this study consisted of those that presented oral candidiasis or other oral conditions, hyposalivation, anemia, hypovitaminosis, diabetes, Sjögren's syndrome, malignant head and neck neoplasia under treatment, or a history of chemo- or radiation therapy. The local research ethics committee approved this study (FOUSP137/11 – February 2012). The patients recruited were enlightened regarding the objectives of the study, and the need for the sample. This type of approach has also been applied in similar, previously published studies,[6,18] and was recently suggested by an international consensus on BMS diagnosis.[25] Patients who agreed to donate a sample of oral mucosa to this study signed an informed consent. This study follows the STROBE-ME statement for observational studies in molecular epidemiology.[26]

Mucosa samples were collected under local anesthesia by punch biopsy (5 mm diameter X 5 mm deep) of the tongue dorsum affected by the burning symptom in the case group. The samples for the control group were obtained from clinically normal tongue dorsum after excision of the fibrous hyperplasia from 5 patients from this group. All the samples were immediately frozen in liquid nitrogen, and stored in a freezer at 80°C, awaiting further laboratory procedures.

Na_v gene expression analysis

Total RNA was extracted from frozen homogenized biopsy specimens with TRIzol™

Reagent (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Next, the samples were treated with DNase I (Invitrogen, Carlsbad, USA) to avoid genomic DNA contamination. The RNA quality was evaluated in 1% agarose gel and NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA), considering 1.8 as a suitable A260/A280 ratio. One microgram of the RNA obtained was submitted to reverse transcription with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions.

Real-time qRT-PCR was performed using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA), and SYBR green fluorescence. Primer pairs of the Na_v genes studied (Table 1) were designed for conducting quantitative assays using the Primer-Blast web tool from the National Center for Biotechnology Information (NCBI).[27] All PCR reactions were performed in triplicate using a total volume of 10µL, which contained a diluted cDNA sample (1:10), primers (400 nM), and SYBR Green qPCR MasterMix (Applied Biosystems, Foster City, USA). Thermal cycling comprised 40 amplification cycles of denaturation at 95°C for 10 s, and an annealing/extension at 60°C for 1 min. Dissociation curve analysis was performed at the end of the 40 cycles, in order to check the identity of the PCR product. The data were normalized by the expression of an endogenous control (Glyceraldehyde 3-phosphate dehydrogenase - GAPDH), and the relative quantification was evaluated by the 2^ (-Delta Delta CT) method.

The differential expression levels of the genes studied in the case and control groups were analyzed by the unpaired t-test or the Mann-Whitney test, depending on the distribution of this data according to adherence to normal distribution (Shapiro-Wilk test, $p > 0.05$). Possible correlations between the clinical and gene expression data were analyzed by the Pearson or the Spearman correlation test, depending on the distribution of data adherence to normal distribution (Shapiro-Wilk test, $p > 0.05$). All the analyses were performed using GraphPad Prism Software version 9.0.2 for Windows (GraphPad Software, San Diego, USA), and the results were considered statistically significantly when $p < 0.05$.

Results

The patients in the BMS group were diagnosed according to the IASP criteria;[1] their clinical characteristics are summarized in Table 2. Accordingly, none had local alterations such as candidiasis or hyposalivation (these characteristics were assessed by exfoliative cytology and sialometry), history of trauma, or previous surgery in the region of the complaint. They also did not present any systemic conditions related to burning mouth symptoms, such as hypovitaminosis, anemia, or uncontrolled or undiagnosed diabetes. Three patients were hypertensive, and were treated with angiotensin-converting-enzyme (ACE) inhibitors, but they did not associate the symptom with the use of these drugs, nor did the symptoms improve after the ACE inhibitors were replaced by other drugs. One third of

Table 1. Set of primers designed for real time PCR reactions.

Gene	Gene bank	TM	Sequence	Size*
$Na_v1.7$	NM_002977.3	58°C	Forward: AATCAGTCACCACTCAGCATT Reverse: CCCCTTCTGCTCTCATTGTC	161bp
$Na_v1.8$	NM_006514.3	61°C	Forward: TGGCAGATGACCTGGAAGAACC Reverse: CGATACGGTAGCAAGTCTTGCG	135bp
$Na_v1.9$	NM_001287223.1	58°C	Forward: CCCAGCAGCTGTTAAAGGAG Reverse: ATTCTGGGACAGTCGTTTGG	242bp
GAPDH	NM_002046	59 °C	Forward: GCATCCTGGGCTACACTGA Reverse: CCACCACCCTGTTGCTGTA	162bp

*bp: base pairs

Table 2. Clinical characteristics of study patients with burning mouth syndrome.

Variable	n (%)
Sex	
Female	12 (100)
Male	0
Age (years)	
Mean	61.4
Range	48–71
Underlying medical condition and related drug	
Hypertension – ACE inhibitors	3 (25.0)
Depression/Anxiety – antidepressants (fluoxetine, sertraline)	4 (33.4)
Hypothyroidism – levothyroxine	3 (25.0)
Osteoporosis – calcium and vitamin D supplementation	1 (8.4)
Burning symptom location	
Apex of the tongue	12 (100)
Lips	2 (16.7)
Gingiva/ Alveolar Ridge	2 (16.7)
Hard palate	1 (8.4)
Burning symptom duration (years)	
Mean	5.4
Range	0.5–27

the BMS patients already had a diagnosis of anxiety and/or depression, and were undergoing some type of treatment for these psychological disorders.

All the patients had symptoms on apex of the tongue, and 9 of these had symptoms only in this region. The other 3 reported a burning complaint in other regions as well, such as the hard palate, gingiva, and lips. The duration of the symptoms ranged from 6 months to 27 years.

There was no statistical difference in age distribution between the BMS and healthy control groups (unpaired t-test, $p = 0.62$), thus they were considered proportionally matched (2.4:1). One sample of the BMS group was excluded because it did not attain the RNA quality required by the spectrophotometric analysis to perform the real-time RT-PCR experiment.

The relative quantification evaluated by the comparative CT method showed an overexpression of $Na_v1.7$ (fold-change = 3.13) gene, and an underexpression of $Na_v1.9$ (fold-change = 0.45) gene, as shown in Figure. However, there were no statistically significant differences between the BMS and healthy control groups regarding the expression of evaluated genes (unpaired t-test $Na_v1.7$: $p = 0.52$ and $Na_v1.9$: $p = 0.36$). The expression of the $Na_v1.8$ gene was not detected

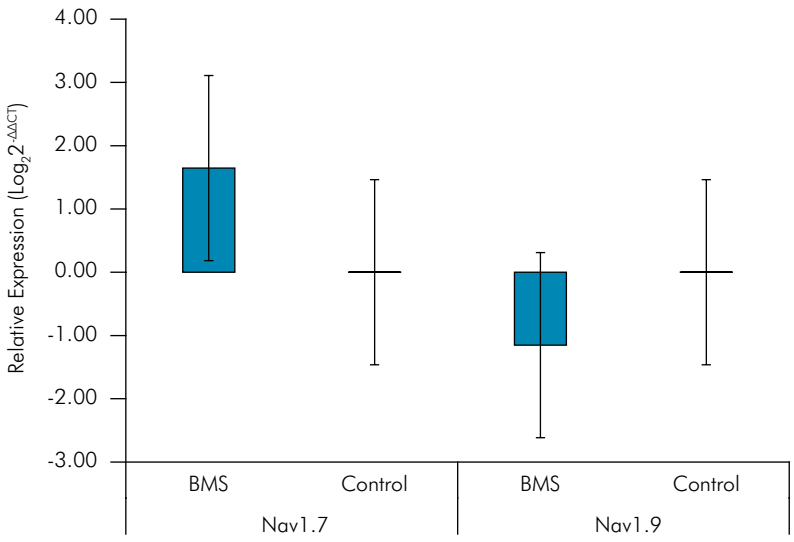

Figure. Differential expression of $Na_v1.7$ and $Na_v1.9$ in BMS patients in relation to healthy patients. The comparative CT method resulted in overexpression of the $Na_v1.7$ (fold-change = 3.13) gene, and underexpression of the $Na_v1.9$ (fold-change = 0.45) gene. However, these differences were not statistically significant, compared with the healthy control group ($p > 0.05$).

in any sample analyzed. No gene expression was correlated with the BMS patients' age, or duration of the burning symptoms (Spearman correlation test, $p > 0.05$).

Discussion

The clinical characterization of the BMS patients who were included in this case-control study corresponds to the epidemiological findings described in the literature.[1,2] BMS causes significant impairment to the patient's quality of life.[2,3] Moreover, since it is a disease with no visible clinical signs or specific diagnostic tests, the patient often feels discredited by family members and health professionals, who are unfamiliar with this condition. Consequently, patients face a great challenge before the final diagnosis is rendered. Most of the time, they are referred to different health specialties, and submitted to different therapies that are mostly ineffective. Thus, scientific research is essential in regard to both the therapeutic and etiological aspects of BMS.[9]

This study focused on the gene expression analysis of voltage-gated sodium channels $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ through real-time RT-PCR. An overexpression was observed in the RNA messenger of $Na_v1.7$, regarding the relation of BMS to the control group [three times higher], and a slight underexpression in that of $Na_v1.9$. However, these differences in the gene expression of $Na_v1.7$ and $Na_v1.9$ were not statistically significant in comparison with the expression of the control group.

Only two studies published in the literature[6,18] have been dedicated to investigating voltage-gated sodium channels in BMS. Concerning $Na_v1.7$, our study depicted results similar to those of other authors, such as Beneng et al.,[18] who also observed no statistically significant differences in the immunohistochemical staining expression of this voltage-gated sodium channel, between the BMS samples and the healthy controls, although they perceived a more intense staining in the BMS samples.

Regarding $Na_v1.8$, no detectable expression of its gene was observed in the present study, in any evaluated sample of either group; moreover, all the samples had high RNA quality, as well as an exponential amplification of the constitutive gene. Yilmaz et al.[6] perceived a slight increase in $Na_v1.8$ immunohistochemical staining in intraepithelial nociceptive nerve fibers of BMS patients, but it was of no statistical difference from that of the healthy control group. In addition, they reported that the immunohistochemical staining of $Na_v1.8$ in intraepithelial nerve fibers was rare or even nonexistent, in contrast with the staining of nerve fibers in deeper layers of the tongue. This may explain the non-detection of $Na_v1.8$ in the present study; that is to say, the small sample specimen may not have attained a detectable level of expression of the $Na_v1.8$ gene in real time RT-PCR analysis.

The investigations by Yilmaz et al.[6] and Beneng et al.[18] researched the protein levels of voltage-gated sodium channels, whereas those of the present study focused on analyzing the gene expression of these channels. Nonetheless, the results of both studies were comparable, especially those of Nav1.7. This may infer that alterations in the post-transcriptional process or during the translation of the messenger RNA of voltage-gated sodium channels are not relevant for determining the BMS pain mechanism.

$Na_v1.9$ has not been previously evaluated in BMS studies published in the literature. A study on lingual neuromas[17] found $Na_v1.9$ by immunohistochemical evaluation, expressed in both painful and asymptomatic neuromas. In the present case-control study, the $Na_v1.9$ gene expression showed a slight decrease in the BMS samples, which may be expected, since neuromas are mainly characterized by a prominent proliferation of neural fibers.

Voltage-gated sodium channels are associated with the generation and conduction of action potentials throughout the nociceptive nerve fiber, resulting in a painful sensation. This pivotal role has made them attractive targets for new therapeutic approaches to pain control,[22] given that they are targeted by a wide variety of local anesthetic, antiarrhythmic, anticonvulsant, and antidepressant drugs.[23]

Although no new drug has been developed with this capacity, this finding has allowed the mechanisms of several neuropathic diseases to be better understood. Recent findings in animal studies concluded that avoiding the SUMOylation of the

collapsin response mediator protein-2 (CRMP-2) – a regulator of the $Na_v1.7$ function – was enough to reverse mechanical allodynia in rats with neuropathic pain. Moreover, female CRMP-2 knock-in mice had reduced $Na_v1.7$ membrane localization and function in sensory neurons, pointing to a possible sex-dependent mechanism.[24]

To date, no description has been reported of changes in the Na_v gene expression profile due to any drug that may interact with these channels, considering that drugs are usually involved in the treatment of depression, and represent a frequent morbidity of BMS patients. An altered genetic expression is usually related to DNA mutations, as found in many channelopathies.[23] Gain-of-function mutations in the $Na_v1.7$ gene have been associated with inherited pain syndromes, while loss-of-function mutations in the same gene have been linked to complete insensitivity to pain.[19-21]

Mutations in voltage-gated sodium channel genes $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ have been related to a hyperexcitable dorsal root ganglion response.[20] Both the mutation of the voltage-gated sodium channels that increase the excitability of the central nervous systems[20], and the peripheral nociceptive activation via accessory molecules of the voltage-gated sodium channels[24] are important to determine the pathophysiology of chronic neuropathic pain. This relationship (mutation vs. activation) is completely unknown in BMS, but it reinforces some characteristics of this disease, such as its higher frequency in female patients, and the possibility of a predominant central or peripheral component.

Conclusion

Although the differences between the BMS patient gene expression of $Na_v1.7$ and $Na_v1.9$ and the gene expression of the control group were not statistically significant, and the $Na_v1.8$ expression was not detected, the functionality of these receptors in BMS was not identified, suggesting that further research is needed regarding voltage-gated sodium channels.

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