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Changes in P2X4 receptor expression following AbobotulinumtoxinA treatment in patients with chronic migraine: A case series study

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Keywords
Chronic Migraine; Medication Overuse Headache; AbobotulinumtoxinA; Gene Expression; NTRK2; Case Series

Abstract

Background: Chronic migraine (CM) is a debilitating neurological disorder often complicated by medication overuse (MO). While abobotulinumtoxinA (ABO-BoNT-A) is a well-established preventive treatment for CM, its molecular mechanisms are not fully understood. Emerging evidence suggests that neurotrophic tyrosine receptor kinase 2 (NTRK2), SRC kinase signaling inhibitor 1 (SRCIN1), and P2X4 purinergic receptor (P2X4R) are involved in migraine chronification and botulinum neurotoxin A (BoNT-A) function, but their roles in humans remain underexplored. This case series investigated changes in NTRK2, SRCIN1, and P2X4R gene expression in peripheral blood pre- and post-BoNT-A treatment and assessed associated clinical outcomes in patients.

Methods: The messenger ribonucleic acid (mRNA) levels of NTRK2, SRCIN1, and P2X4R genes were analyzed in a sample of eight patients with CM and MO following BoNT-A treatment using quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, migraine characteristics were assessed using Migraine Disability Assessment Scale (MIDAS), Headache Impact Test-6 (HIT-6), and Patient Health Questionnaire-9 (PHQ-9).

Results: The intervention resulted in early measurable improvements in migraine symptoms and disability. Post-treatment, P2X4R expression significantly increased ($P < 0.05$), while NTRK2 and SRCIN1 showed no significant changes.

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Conclusion: Findings indicate that P2X4R upregulation may be linked to the therapeutic effects of BoNT-A, while NTRK2 and SRCIN1 appear uninvolved. The trend in P2X4R expression suggests it may serve as a predictive biomarker and therapeutic target, but further validation in larger cohorts is necessary.

Introduction

Migraine is a debilitating neurological disorder identified as a leading cause of disability-adjusted life years (DALYs), according to the 2021 Global Burden of Neurological Diseases (GBD) study.[1]

Approximately, 15% of the world's population and around 14% of people in Iran are affected, with about 3% of episodic migraines progressing to chronic migraine (CM) each year.[2-4] This process, known as chronification, is linked to changes in the brain's neural and chemical systems, which lead to increased sensitivity and inflammation.[5] Botulinum neurotoxin type A (BoNT-A) is used as a preventive treatment for CM. Its mechanism of action involves cleaving SNARE proteins, which disrupts the release of neurotransmitters.[6,7] Recent clinical trials highlight the important role of cellular signaling pathways and specific gene expressions in mediating the effects of BoNT-A.[8]

Many factors were studied independently in migraine chronification and the mechanism of action of botulinum toxin. Neurotrophic tyrosine receptor kinase 2 (NTRK2), SRC family kinases (SFKs) inhibitors, and the P2X4 purinergic receptor (P2X4R) were common among all.[9-15]

NTRK2 encodes the tropomyosin receptor kinase B (TrkB) receptor, which is responsible for mediating signals from brain-derived neurotrophic factor (BDNF).[16] The TrkB receptor is crucial for several processes, including synaptic plasticity, calcium signaling, and neuroinflammation. When NTRK2 signaling is dysregulated, it can aggravate pain pathways and increase neuronal excitability. This can lead to the development of migraine chronicity and heightened sensitivity in CMs.[17,18]

Previous result showed that SFK inhibitors, such as SRC kinase signaling inhibitor 1 (SRCIN1) – which encodes a key negative regulator of SFKs – impaired intracellular signaling and cellular migration. These inhibitors have a synergistic antagonistic effect against BoNT-A by disrupting phosphorylation-dependent signaling pathways that are crucial for the toxin's activity and stability.[14].Dysregulation of SRCIN1 may enhance neuronal signaling and interact with neurotrophic factors, such as BDNF. Impaired communication between calcitonin gene-related peptide (CGRP) and cytokines leads to the maintenance of trigeminal ganglion (TG) sensitivity and sensitization of the trigeminovascular system, contributing to migraines' progression.[11,15]

The P2X4R is crucial in neuroinflammation and pain mechanisms.[19] Its activation leads to the release of inflammatory mediators and contributes to sensitization in migraines.[10]

Molecular docking analyses suggest that multiple types of interactions – such as hydrophobic, electrostatic, and hydrogen bonds – occur between the residues of the botulinum toxin light chain and the B chain of the purinergic receptor P2X4. Research conducted on microglial cells has shown that botulinum toxin binds to the P2X4R with high affinity, resulting in the receptor's proteolysis. This process subsequently leads to the inhibition of the p38 mitogen-activated protein kinases (p38-MAPKs) signaling pathway activated by P2X4R.[12]

This study aims to investigate the complex molecular and genetic interactions between botulinum neurotoxin (BoNT) and key neuronal pathways, specifically focusing on the roles of the NTRK2, SRCIN1, and P2X4R genes. It represents one of the first investigations into changes in candidate gene expression following abobotulinumtoxinA (ABO-BoNT-A) treatment in patients with CM. This hypothesis-generating study offers initial insights pointing to a possible role for purinergic signaling in the efficacy of BoNT-A and its potential therapeutic strategies.

Materials and Methods

Study design and population: This molecular analytical case-series study evaluated gene expression changes in eight cases of CM with medication overuse headache (MOH) in adults who were treated with BoNT-A at the headache clinic of Sina Hospital in Tehran City, Iran, between September 2019 and June 2020. This study was approved by the Research Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.NI.REC.1400.011), and written informed consents were obtained from all patients.

The study protocol consisted of four monthly visits, including one screening visit (visit 1) followed by three subsequent visits after the injection (visits 0 to +3) for clinical assessment, and headache characteristics were recorded. In parallel, gene expression assessments were conducted at two time points: at visit 0 and again 6 to 8 weeks

after the injection. Participants with confirmed diagnosis of CM + MOH as per the diagnostic criteria outlined in the third edition of the International Classification of Headache Disorders (ICHD-3) whose attacks were not controlled by first-line medications and met the eligibility criteria were enrolled during the baseline screening period. From four weeks before the injection (weeks -4 to 0), patients received instructions on how to fill out a paper-based headache diary. At the initial visit (visit 0), patients received Dysport® injection after the first sample was collected and were required to maintain a daily headache diary for the entire duration of the study.

Exclusion criteria were established to minimize confounding factors. By excluding participants with significant comorbidities, such as severe systemic diseases, renal, hepatic, or neurological disorders, and those who had recent exposure to similar treatments, the study aimed to provide a reliable assessment of molecular changes and valuable insights into the underlying mechanisms in response to BoNT-A treatment. Additionally, pregnant or breastfeeding women, as well as those intending to become pregnant during the study period, were excluded.

Demographic and anthropometric data: After fulfilling the inclusion and exclusion criteria and obtaining informed consent from participants, a structured interview was performed by the researcher. At the baseline visit, demographic data, medications, and information about history of migraine and CM onset were collected from all subjects. Body weight was measured on a medical scale, and height was measured using a standard stadiometer. Body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m^2).

At the baseline visit, demographic data, medications, and information about history of migraine, CM onset and other disorders was collected from all subjects.

Headache diaries and clinical assessments: The migraine characteristics were recorded using a paper-based diary designed by the lead researcher, Dr. Togha. This diary included onset time of attacks, duration of attacks, severity of attacks using a visual analog scale (VAS), and the daily number of abortive drugs consumed.[20] Participants completed a baseline diary during the month prior to the start of the intervention. Following this, all patients were asked to complete a headache diary during the study.

Further assessments were conducted using the Migraine Disability Assessment Scale (MIDAS) to evaluate headache-related disability, the Headache Impact Test-6 (HIT-6) to assess quality of life (QOL), and the Patient Health Questionnaire-9 (PHQ-9) to determine the severity of depression at two time points, baseline and three months after the injection. These assessments were performed by the researcher using valid questionnaires.[21]

Intervention: Dysport® in sterile, single-use vials containing 500 international units for reconstitution was used for the preventive treatment of patients. It was administered intramuscularly at a dose of up to 500 units, targeting the corrugator, procerus, superior frontalis, temporalis, splenius capitis, occipitalis, and trapezius muscles, in accordance with the Phase III Research Evaluating Migraine Prophylaxis Therapy (PREEMPT) clinical program.[22] The interventions were performed by a neurologist who specialized in headache and facial pain.

Blood sampling and quantitative real-time polymerase chain reaction (qRT-PCR): Blood (5 ml) was collected from the cubital vein at baseline and again six to eight weeks after the treatment began,[23] which is identified as the peak efficacy period for the effects of BoNT. Total ribonucleic acid (RNA) was extracted from the peripheral blood samples immediately after collection, following standard protocols using the FavorPrep™ RNA Kit. The quality of the extracted RNA was assessed through agarose gel electrophoresis and ultraviolet (UV) spectroscopy.

Complementary deoxyribonucleic acid (cDNA) was synthesized using the Easy cDNA Ultra-TM Synthesis Kit according to the manufacturer's protocol. Primers for all candidate genes were designed using Gene Runner software and subsequently validated on the National Center for Biotechnology Information (NCBI) website, as detailed in table 1. The beta-2-microglobulin (B2M) gene served as a stable endogenous reference gene for normalization, as confirmed by GeNorm and NormFinder analyses.[24]

Agarose gel electrophoresis was conducted to verify the predicted size of the polymerase chain reaction (PCR) amplicons for the genes. Standard curves for each gene were generated using serial dilutions (1:4) of pooled cDNA, which was extracted from blood samples of eight subjects. qRT-PCR was performed using SYBR Green [(2x) SYBR Green Master Mix] on the Applied Biosystems 7500 FAST Dx Real-Time PCR System,

utilizing a triplicate method for qRT-PCR.

Table 1. Sequences of primers used for quantitative real time polymerase chain reaction (qRT-PCR)

Genes primer	Primer sequence
B2M	Forward: AGATGAGTATGCCTGCCGTGT Reverse: TGCGACATCTTCAAACCTCCAT
NTRK2	Forward: CACCTTGACTTGTCTGAACTGAT Reverse: GTCTTGATCCACATAATGTCACAGG
SRCIN1	Forward: GCCTGATGGGGAACGCTC Reverse: GGGTACTCCGCATCGTCC
P2X4R	Forward: CCTGTGCCCCGAGATTCCA Reverse: CCTGCCTGTTGAGACTCCGT

B2M: Beta-2-microglobulin; NTRK2: Neurotrophic tyrosine receptor kinase 2; SRCIN1: SRC kinase signaling inhibitor 1; P2X4R: P2X4 purinergic receptor

Data were analyzed using the SPSS software (version 19, SPSS Inc., Chicago, IL, USA), with a significance level set at $\alpha = 0.05$. The normality of quantitative variables in the study was assessed through various methods, including visual inspections and statistical tests such as the Shapiro-Wilk and Kolmogorov-Smirnov tests. All variables, except for "migraine days at the last visit", "maximum intensity at the baseline visit", and "days of acute treatment use at the last visit", followed a normal distribution. For these exceptions, the differences between values at the last and first visits were calculated, and normality was re-evaluated for these differences. Inferential statistical comparisons were conducted using a paired samples t-test and repeated measures ANOVA for normally-distributed quantitative variables to compare measurement values at different time points. Qualitative variables were reported as frequencies and percentages, with the chi-square test used for comparisons of categorical data over time. We calculated 95% confidence intervals (CIs) for all primary outcomes and computed effect sizes to assess the magnitude of observed differences. Additionally, we made necessary adjustments for multiple comparisons to control the family-wise error rate.

Results

Participant characteristics: Eight patients with CM without aura and MOH, with average age of 35.62 [standard deviation (SD) = 13.60], mostly women (75%), were enrolled in our study. Additionally, five patients reported a family history of migraines on the maternal side. All received botulinum toxin injections (Table 2).

Table 2. Demographic, clinical, and medication characteristics of the participants

Characteristics	Value
Age (year) (mean ± SD)	35.62 ± 13.60
BMI (kg/m^2) (mean ± SD)	24.79 ± 3.22
Migraine onset age (year) (mean ± SD)	22.75 ± 12.18
CM onset age (year) (mean ± SD)	31.87 ± 15.64
Gender/women [n (%)]	6 (75.0)

BMI: Body mass index; CM: Chronic migraine; SD: Standard deviation

Regarding comorbidities, some individuals had conditions such as restless legs syndrome (RLS), asthma, constipation, anxiety, and gastroesophageal reflux disease (GERD), while others had no significant comorbidities. The concurrent prophylactic trials used by participants included antidepressants, antiepileptics, and supplements. Acute medications varied among participants and included acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs), combined analgesics, triptans, and ergotamine.

Clinical outcomes in migraine characteristics and its disability: The number of migraine days per month significantly decreased from a mean of 23.13 (SD = 5.94) at baseline to 11.88 (SD = 9.70) at the interim assessment, and then to 14.25 (SD = 6.92) at the final visit ($P = 0.003$). Additionally, the maximum migraine intensity also decreased significantly over time, dropping from a mean of 9.00 (SD = 1.31) at baseline to 6.25 (SD = 1.49) at the interim visit ($P < 0.001$). In contrast, the minimum intensity showed no significant change across the time points ($P = 0.890$). While the mean duration of migraine attacks exhibited a decreasing trend – from 28.63 hours (SD = 21.98) at baseline to 13.25 hours (SD = 9.19) at the interim visit and 15.63 hours (SD = 15.89) at the final visit – this difference did not reach statistical significance ($P = 0.143$). Furthermore, the number of days with acute headache medication use showed a significant reduction, decreasing from a mean of 20.75 (SD = 6.11) at baseline to 7.25 (SD = 7.72) at the interim assessment and 13.50 (SD = 7.45) at the final visit ($P < 0.001$). Significant differences were observed in post hoc comparisons between baseline/interim and baseline/final (Tables 2, 3).

Table 3. The differences of migraine characteristics between tree time-points

	Baseline visit	Interim visit	Final visit	P-value time effect within group differences*	Post hoc**
Migraine (days per month) (mean ± SD)	23.13 ± 5.94	11.88 ± 9.70	14.25 ± 6.92	0.003	Baseline/interim, baseline/final
Migraine attacks duration (hour) (mean ± SD)	28.63 ± 21.98	13.25 ± 9.19	15.63 ± 15.89	0.143	
Maximum intensity (mean ± SD)	9.00 ± 1.31	6.25 ± 1.49	7.88 ± 2.17	< 0.001	Baseline/interim
Minimum intensity (mean ± SD)	4.63 ± 2.07	3.25 ± 1.16	3.88 ± 1.46	0.890	
Acute medication (day) (mean ± SD)	20.75 ± 6.11	7.25 ± 7.72	13.50 ± 7.45	< 0.001	Baseline/interim, baseline/final

*Repeated measures analysis of variance (ANOVA); **Bonferroni
SD: Standard deviation

Headache-related disability was assessed by HIT-6, PHQ-9, and MIDAS scores, which indicated reduced headache impact and improved daily functioning, from baseline to last visit. The HIT-6 and PHQ-9 scores showed statistically significant improvements. The HIT-6 score decreased significantly from 67.88 (SD = 3.72) at baseline to 61.88 (SD = 6.49) at the final visit (P = 0.019), indicating a significant reduction in headache-related impact. Similarly, the PHQ-9 score, which reflects depressive symptoms, showed a significant reduction from 11.25 (SD = 5.37) to 9.38 (SD = 5.15) (P = 0.040). The MIDAS score also decreased numerically – from 65.75 (SD = 45.61) to 44.38 (SD = 36.00) – but this reduction was not statistically significant (P = 0.175) (Table 4).

Gene expression results: Total RNA was extracted from peripheral blood samples, with agarose gel electrophoresis revealing intact bands and UV spectrophotometry indicating RNA purity (A260/A280 ratios between 1.8 and 2.0). cDNA synthesis was confirmed through PCR amplification, showing expected amplicon sizes that validated primer specificity. The B2M gene was consistently expressed across samples, establishing it as a reliable housekeeping gene for normalization. All qRT-PCR reactions were conducted in triplicate and demonstrated high linearity, with R^2 values exceeding 0.99. The presence of a single peak in the melting curve further confirms the specificity of the amplified products and the overall accuracy of the real-time PCR (RT-PCR) assay. Among the genes studied, P2X4R displayed a significant upregulation with a relative expression level of 4.619 (P = 0.003), but the wide 95% CI (0.775-18.103) indicates potential instability due to the small sample size. In contrast, NTRK2 and SRCIN1 showed no significant changes in expression levels, with relative expressions of 1.408 (P = 0.508) and 1.515 (P = 0.306), respectively, and broad CIs encompassing 1.

Discussion

The P2X4R has been identified as a susceptibility gene in CM. Previous studies indicate that its expression in microglia contributes to neuropathic and inflammatory pain by activating the p38-MAPK pathway.[25,26]

Table 4. The differences in headache-related disability from baseline to final visit

	Mean ± SD	95% CI of the difference		Cohen's d	95% CI		P*
		Lower	Upper		Lower	Upper	
MIDAS score final visit	44.38 ± 36.00						
MIDAS score baseline visit	65.75 ± 45.61						
Change	-21.38 ± 40.06	-54.87	12.12	-0.534	-1.26	0.23	0.175
HIT-6 score final visit	61.88 ± 6.49						
HIT-6 score baseline visit	67.88 ± 3.72						
Change	-6.00 ± 5.61	-10.69	-1.31	-1.070	-1.93	-0.16	0.019
PHQ-9 score final visit	9.38 ± 5.15						
PHQ-9 score baseline visit	11.25 ± 5.37						
Change	-1.88 ± 2.10	-3.63	-0.12	-0.893	-1.70	-0.04	0.040

*Using paired samples t-test
MIDAS: Migraine Disability Assessment Scale; HIT-6: Headache Impact Test-6; PHQ-9: Patient Health Questionnaire-9; CI: Confidence interval; SD: Standard deviation

Additionally, microglial cells molecular docking studies have reported that botulinum toxin may induce proteolysis of the P2X4R through high-affinity molecular binding, potentially inhibiting the P2X4R-p38MAPK signaling pathway.[12]

This exploratory case series is one of the first studies to investigate changes in the expression of NTRK2, SRCIN1, and P2X4R genes in response to ABO-BoNT-A treatment in patients with CM. Clinically, despite variations in long-term efficacy among patients, BoNT-A treatment has been associated with reductions in migraine frequency, severity, duration, and acute medication use. It also improves disability, QOL, and depressive symptoms, consistent with previous reports.[27,28] In the molecular preliminary results, we observed an unexpected and significant approximately 4.6-fold upregulation of P2X4R gene expression ($P = 0.003$) following BoNT-A treatment. This upregulation showed the molecular effects of BoNT-A and the complexity of CM pathophysiology. It suggests that BoNT-A may influence neuroinflammatory pathways that play a central role in the development of migraines. The increase in P2X4R expression following BoNT treatment might serve as a compensatory mechanism in response to the proteolysis and subsequent depletion of receptor proteins. In essence, the proteolytic effect of BoNT-A may trigger a feedback loop that results in heightened expression of P2X4R to offset the loss of these receptor proteins. This upregulation further supports the hypothesis that neuroinflammation and pain pathways are involved in the modulation of migraine attacks. P2X4R is a critical receptor implicated in both peripheral and central pain mechanisms. The administration of BoNT highlights the potential for its analgesic effects to occur through the regulation of neuroinflammatory pathways. Preliminary findings suggest that the P2X4R gene could be a promising candidate for studying the effects of BoNT. However, it is important to interpret the significant increase in P2X4R gene expression following treatment with caution, given that this study lacked a control group and involved a small sample size.

Unlike the promising results with P2X4R, no significant changes were observed in the expression of NTRK2 (TrkB) and SRCIN1. This stability may indicate that these genes are less closely linked to the immediate effects of BoNT or do not represent a primary response to it. Alternatively, delayed transcriptional responses or individual genetic variability could explain these findings. However, previous in vitro studies have shown that SRCIN1, known to inhibit SFKs, and NTRK2, a membrane-bound kinase, are involved in neuronal signaling following exposure to BoNT. Specifically, microarray transcriptomic profiling of SH-SY5Y cells treated with BoNT-A for 48 hours revealed changes in the expression levels of SRCIN1 and TrkB. Additionally, SRCIN1 has demonstrated an antagonistic effect against various serotypes of botulinum toxin.[13,14] Therefore, confirming these changes in expression and the role of these factors may extend beyond the time frame of this study, highlighting the need for longer follow-up periods in clinical investigations to explore these mechanisms more thoroughly. Additionally, the relatively short duration of treatment may have limited the ability to detect more subtle or delayed changes in gene expression. Larger studies with longer follow-up periods are needed to fully explore the potential role of NTRK2 and SRCIN1 in migraine BoNT treatment.

Strengths and weaknesses of the study design: Unlike study limitations including small size of the sample and lack of control group, this research provides valuable insights into the therapeutic mechanism of BoNT-A, suggesting that it may influence purinergic signaling in CM. These findings highlight the importance of targeting these pathways for therapeutic innovation and long-term effects.

Broader implications and future directions: The unexpected upregulation of P2X4R highlights the need to re-evaluate how BoNT-A influences molecular mechanisms in both central and peripheral purinergic systems. Future studies involving larger-scale and case-control cohorts are essential to validate the relationship between BoNT-A administration, P2X4R expression, and clinical outcomes. Additionally, expanding the analysis to include a broad transcriptome-wide approach could reveal additional molecular pathways and targets relevant to the BoNT-A effects. In particular, comprehensive gene expression profiling may help elucidate the functional significance of observed changes in P2X4R upregulation and clarify its potential role in migraine pathophysiology. Investigating the interactions between P2X4R and other molecular targets in CM could lead to the discovery of novel therapeutic strategies.

Conclusion

Based on our exploratory case series, we propose

that BoNT-A may be involved in purinergic signaling and microglia-mediated neuroinflammation through P2X4R in CM. These initial findings generate new hypotheses and indicate a promising direction for future research into novel therapeutic options targeting these pathways for migraine treatment. However, given the small sample size and exploratory nature of the study, these results should be interpreted with caution. Further investigation in larger, controlled studies is necessary to confirm the observed associations and clarify the mechanistic role of P2X4R in response to BoNT-A treatment.

Conflict of Interests

The authors declare no conflict of interest in this study.

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