

Relationship between toll-like receptor expression in the distal facial nerve and facial nerve recovery after injury

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Hye Kyu Min¹, In Hyeok Kim¹, Jae Min Lee¹, Junyang Jung²,
Hwa Sung Rim¹, Dae Woong Kang¹, Sang Hoon Kim¹, Seung
Geun Yeo¹ 

Abstract

Objectives: This study aimed to determine whether toll-like receptor expression patterns differ in the distal facial nerve during recovery after crushing and cutting injuries. **Methods:** Adult male Sprague-Dawley rats underwent crushing or cutting injury of the unilateral facial nerve. Their whisker movement and blink reflex were examined. Western blotting was performed with the normal nerve on the left side and the damaged nerve on the right side, four days, 14 days, and 3 months after injury. **Results:** The scores of whisker movements and blink reflex in the crushing group showed improvements, while the score of the cutting group was significantly lower at 14 days and 3 months ($p < 0.05$). Western blotting showed that TLRs 11 and 13 increased in the crushing group, and TLRs 1, 2, 3, 4, 5, 8, 10, 11, 12, and 13 increased in the cutting group after 14 days ($p < 0.05$). After 3 months, TLRs 10 and 11 increased in the crushing group, and TLRs 1, 4, 5, 8, 11, and 12 increased in the cutting group ($p < 0.05$). **Conclusion:** TLRs 1, 4, 5, 8, and 12 are related to nerve degeneration after facial nerve injury, and TLRs 10, 11, and 13 are related to recovery from facial palsy.

Keywords

facial palsy, crushing, cutting, nerve injury, toll-like receptor

Introduction

Pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs), mediate the innate immune response by initially detecting pathogens in mammals. PRRs trigger intracellular signaling cascades that lead to the transcriptional expression of inflammatory mediators that coordinate the clearance of invading pathogens and infected cells.^{1,2}

A number of studies on the role of PRRs in various diseases related to the innate immune response, especially TLRs, are actively underway. TLRs were first discovered as receptors involved in dorsal-ventral patterning during the development of *Drosophila melanogaster*. To date, 10 types of

TLRs have been identified in humans and at least 13 in mice. Each TLR recognizes different pathogen-associated molecular patterns (PAMPs), such as double-stranded DNA (CpG), single-stranded ribonucleic acid (ssRNA), lipoproteins, lipopolysaccharide (LPS), and flagellin, depending on the site in which they exist. TLRs are expressed in various immune system-related cells, such as B cells,

¹Department of Otolaryngology - Head & Neck Surgery, School of Medicine, Kyung Hee University, Seoul, South Korea

²School of Medicine, Kyung Hee University, Seoul, South Korea

Corresponding author:

Seung Geun Yeo, Department of Otolaryngology-Head and Neck Surgery, Kyung Hee University Medical Center, 23 Kyungheedaero, Dongdaemun-gu, Seoul 02447, South Korea.

Email: yeo2park@gmail.com



T cells, macrophages, and dendritic cells. TLRs 3, 7, 8, and 9 are present within the endoplasmic reticulum and endosomal membrane to recognize the nucleic acid of the pathogen. TLRs 1, 2, 4, 5, and 6 are present on the cell membrane.³⁻⁵

It has been reported that TLRs are also present in the cells of the nervous system, such as microglia, astrocytes, oligodendrocytes, neurons, and neuronal progenitor cells, and play an important role in the damage and regeneration of peripheral and central nervous systems. In addition to pathogen-derived ligands, endogenous TLR ligands, called damage-associated molecular patterns (DAMPs), can activate different TLRs. DAMPs include by-products from brain ischemia or traumatic injury of nerves or amyloid-beta peptides in Alzheimer's disease. Although the role of TLRs activated by DAMPs has not been clearly elucidated, it has been reported to inhibit the proliferation of neural progenitor cells and promote neurodegeneration during nerve damage of the central and peripheral nerves.⁴⁻⁶

Based on these results, TLRs are expected to play an important role in facial palsy, a peripheral nerve disease in the field of otolaryngology. Although facial palsy is not a life-threatening disease, it is important to cure the disease as it can significantly affect the patient's quality of life by deteriorating the psychological, emotional, and social activity if recovery remains incomplete.^{7,8} Although several studies are being conducted that aim to cure patients with various causes of facial paralysis, there are still no biomarkers specific to facial palsy, and the exploration of biological factors affecting nerve regeneration has not been greatly improved.

Therefore, we aimed to explore the biological factors involved in neuro-suppression and nerve regeneration by using an animal model of facial palsy. This study aimed to determine whether TLRs are expressed in the distal facial nerve after crushing and cutting injuries and whether TLR expression patterns differ during recovery from crushing and cutting injuries.

Materials and methods

Animals

Forty-eight six-week-old male Sprague-Dawley (S-D) rats weighing 200–250 g were used according to the guidelines of the animal laboratory of our center. We randomly divided the rats into two

groups: crushing and cutting. Twenty-four rats were subjected to crushing injury and the other 24 to cutting injury of the right facial nerve. Eight rats in each group were sacrificed 4 days after injury, eight in each group were sacrificed 14 days after injury, and 8 rats in each group were sacrificed 3 months after injury (Table 1). Uninjured normal left facial nerves of the 48 S-D rats were used as the control group.

Procedure

All S-D rats were anesthetized using 5% isoflurane (Forane solution, Choongwae, Hwasung, Korea) mixed with 80% oxygen, and anesthesia was maintained with 2% isoflurane. Under inhalation anesthesia, a post-auricular incision was made on the right side, and the mastoid process and parotid gland were identified. The facial nerve trunk and its five branches (temporal, zygomatic, buccal, mandibular, and cervical) were exposed. The facial nerve was pressed for 30 s or completely cut at the midpoint between the site where the facial nerve trunk emerged and the area where the facial nerve branches under Zeiss microscope (Carl Zeiss, Germany). The wound was then sutured. At four days, 14 days, and 3 months after facial nerve injury, the activity of the facial nerve was evaluated by examining the whisker movement of the vibrissae muscle and the blink reflex of the eyelid. In addition, the normal facial nerve on the left side and the distal portion to the injury site of the facial nerve on the right side were collected four days, 14 days, and 3 months after facial nerve injury. Total proteins were extracted from the collected facial nerves, and western blotting was performed. The study protocol was approved by the Clinical Research Ethics Committee.

Vibrissae movement test/eye closure, blinking reflex

The degree of damage to and recovery rate of the facial nerve were assessed by measuring the

Table 1. Number of animals in each experimental group.

	Crushing group (N=24)	Cutting group (N=24)
Fourth day	8	8
14 th day	8	8
Third month	8	8

whisker movement of the vibrissae muscle and blink reflex of the eyelid. Briefly, whisker movement was evaluated by measuring the degree of movement of the whiskers and their reference position when an alcohol container was placed around the nose of an S-D rat to stimulate the sense of smell. The results were scored on a five-point scale: one point, if there was no movement with the whiskers tilted back (no movement, posterior); two points if there was a slight movement with whiskers tilted back (light tremor, posterior); three points if the movement was large with the whiskers tilted back but was less than normal (greater tremor, posterior); four points if whisker movement was at the same level as normal, but was tilted back (normal movement, posterior); and five points if the whiskers showed normal movements and were in a forward position as on the undamaged side (Table 2).^{9,10}

The blink reflex of the eyelids was evaluated based on the degree of narrowing of the eyelid gap when the area around the eye was stimulated with the same intensity of wind using an air pump. The results were scored on a five-point scale: one point if there was no movement at all (no movement), two points if the eyelid moved but did not show narrowing (contraction/no closure); three points if the eyelid gap narrowed $\leq 50\%$ (50% closure); four points if the eyelid gap narrowed $>50\%$ but $\leq 75\%$ (75% closure); and five points if the eyelid closed completely, similar to the

eyelid on the undamaged side (complete closure) (Table 3).^{9,10}

Western blotting

Proteins were extracted from rat facial nerve tissues using radioimmunoprecipitation assay (RIPA) buffer containing a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA, USA). Lysates were clarified by centrifugation, and the supernatants were collected. Equal amounts of protein (25 μg) were fractionated by electrophoresis on 8%–10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. After incubation with 5% nonfat milk in tris-buffered saline (10 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, the membranes were incubated overnight at 4°C with antibodies to TLR 1 (Abcam, ab180798, 1:500), TLR 2 (Abcam, ab213676, 1:500), TLR 3 (Novusbio, NBP2-24,904, 1:500), TLR 4 (LSBio, LS-C663627, 1:1000), TLR 5 (Abcam, ab62460, 1:1000), TLR 6 (Novusbio, NBP1-54,336, 1:500), TLR 7 (LSBio, LS-C806008, 1:1000), TLR 8 (Abcam, ab180610, 1:500), TLR 9 (Abcam, ab134368, 1:500), TLR 10 (LSBio, LS-C314873, 1:200), TLR 11 (Novusbio, NBP100-56,742, 1:1000), TLR 12 (Novusbio, NBP2-24,833, 1:500), TLR 13 (LSBio, LS-C148184, 1:500), and β -actin (Santa Cruz, 47,778, 1:100,000). After washing, the membranes were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated mouse anti-rabbit secondary antibodies for 2 h at room temperature. Blots were developed with enhanced chemiluminescence reagents (Clarity™ Western ECL Substrate, Bio-Rad), and protein bands were quantitated using Image J software (United States National Institutes of Health, MD, USA).

Statistical analysis

All results are expressed as the mean \pm standard error. The differences in the behavioral test scores between each group were analyzed using an independent sample t-test. The levels of expression of TLRs were compared between groups using a one-way analysis of variance (ANOVA) and post-tested using the least significant difference test (LSD). All data were analyzed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). A *p*-value of <0.05 was considered statistically significant.

Table 2. Vibrissae observation scale.

Score	Movement	Position
1	No movement	Posterior
2	Light tremor	Posterior
3	Greater tremor	Posterior
4	Normal movement	Posterior
5	Normal movement	Anterior

Table 3. Scale of eye closing and blinking reflex observation.

Score	Movement
1	No movement
2	Contraction/no closure
3	50% closure
4	75% closure
5	Complete closure

Results

Whisker movement of the vibrissae muscle and blink reflex of the eyelid

Figure 1 The cutting and crushing groups showed significantly lower scores for whisker movement of the vibrissae muscle and blink reflex of the eyelid than the control group 4 days after injury. However, the score of the crushing group was not significantly different from that of the control group at 14 days and 3 months, indicating recovery of the facial nerve function ($p > 0.05$). In contrast, the score of the cutting group was still significantly lower than that of the control group, even after 14 days and 3 months ($p < 0.05$).

Protein expression patterns

Figure 2 Western blotting showed that 4 days after nerve injury, the expression of TLRs 1, 2, 4, 5, 8, 10, 11, 12, and 13 increased in the crushing group, whereas the expression of TLRs 1, 4, 5, 8, 10, 11, 12, and 13 increased in the cutting group ($p < 0.05$). After 14 days, TLR 11 and 13 increased in the crushing group, and TLRs 1, 2, 3, 4, 5, 8, 10, 11, 12, and 13 increased in the cutting group ($p < 0.05$). After 3 months, TLRs 10 and 11 increased in

the crushing group, and TLRs 1, 4, 5, 8, 11, and 12 increased in the cutting group ($p < 0.05$).

Discussion

When a nerve is damaged, its distal portion undergoes a change called Wallerian degeneration. This change usually occurs in axons distal to the site of injury within 24–48 h and continues for about a week, after which the regeneration process starts. We analyzed the expression level of TLRs on the fourth day after the injury, where Wallerian degeneration progressed, and on the 14th day after the injury, where regeneration progresses to observe the differences in TLR expression patterns between degeneration and regeneration processes. After nerve injury, the axon skeleton breaks down, and the axon membrane separates. Temporarily, Schwann cells multiply, followed by axonal degeneration, decomposition of the myelin sheath, and infiltration by macrophages. Macrophages mobilized by Schwann cells remove the debris remaining after degeneration. Myelination is regulated both positively and negatively. Positive regulators that promote myelination include Krox-20 (Egr-2), Sox-10, Oct-6 (SCIP, Tst-1, POU3fl), and NF- κ B, which are transcription factors present in normal

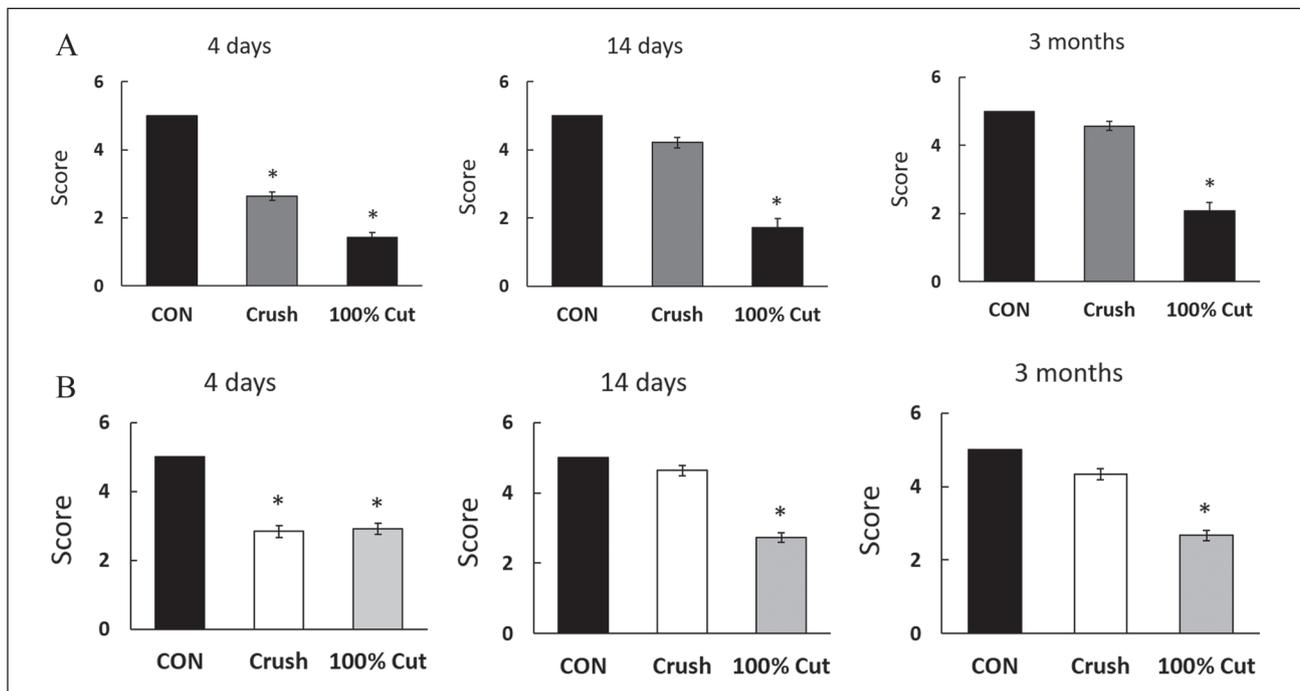


Figure 1. (A) Whisker movements of the vibrissae muscle and (B) Scale of eye closing and blinking reflex in rats at four days, 14 days, and 3 months after facial nerve injury. CON: control group. * $p < 0.05$.

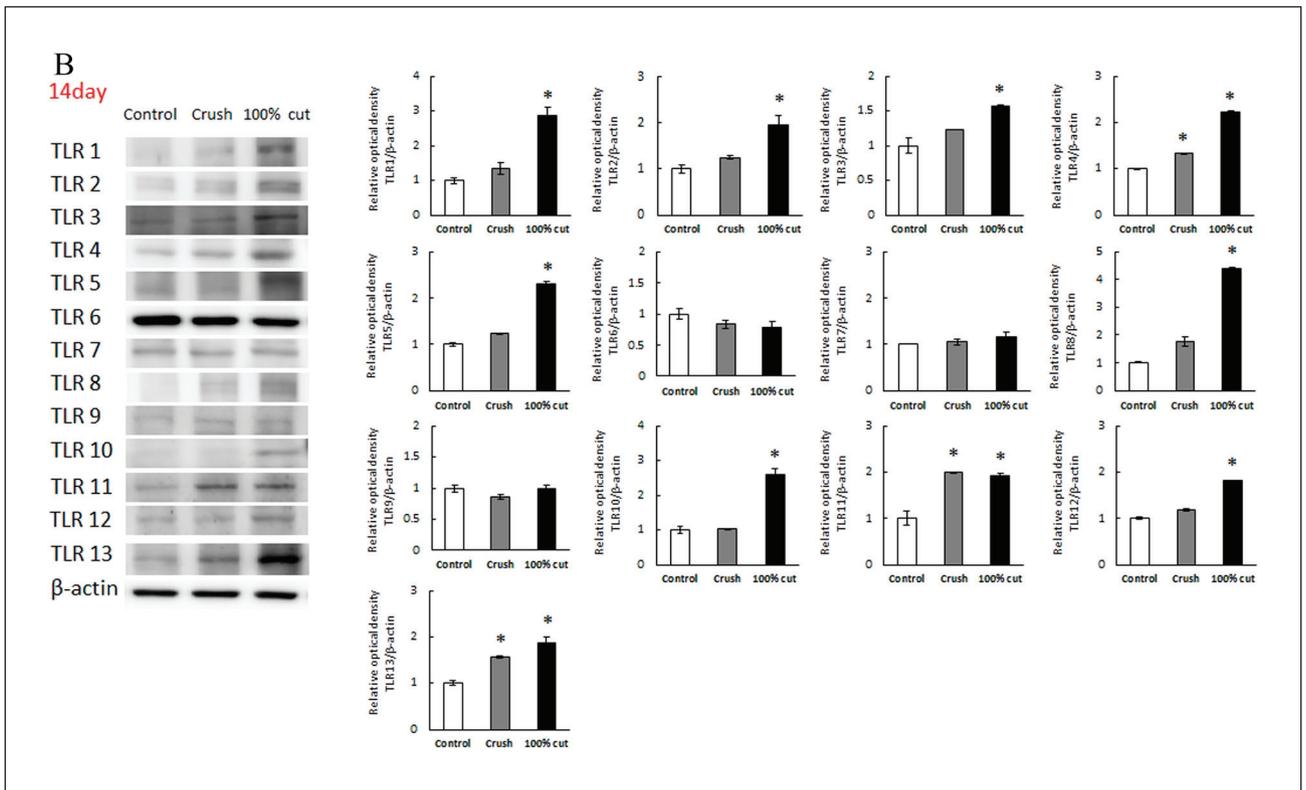
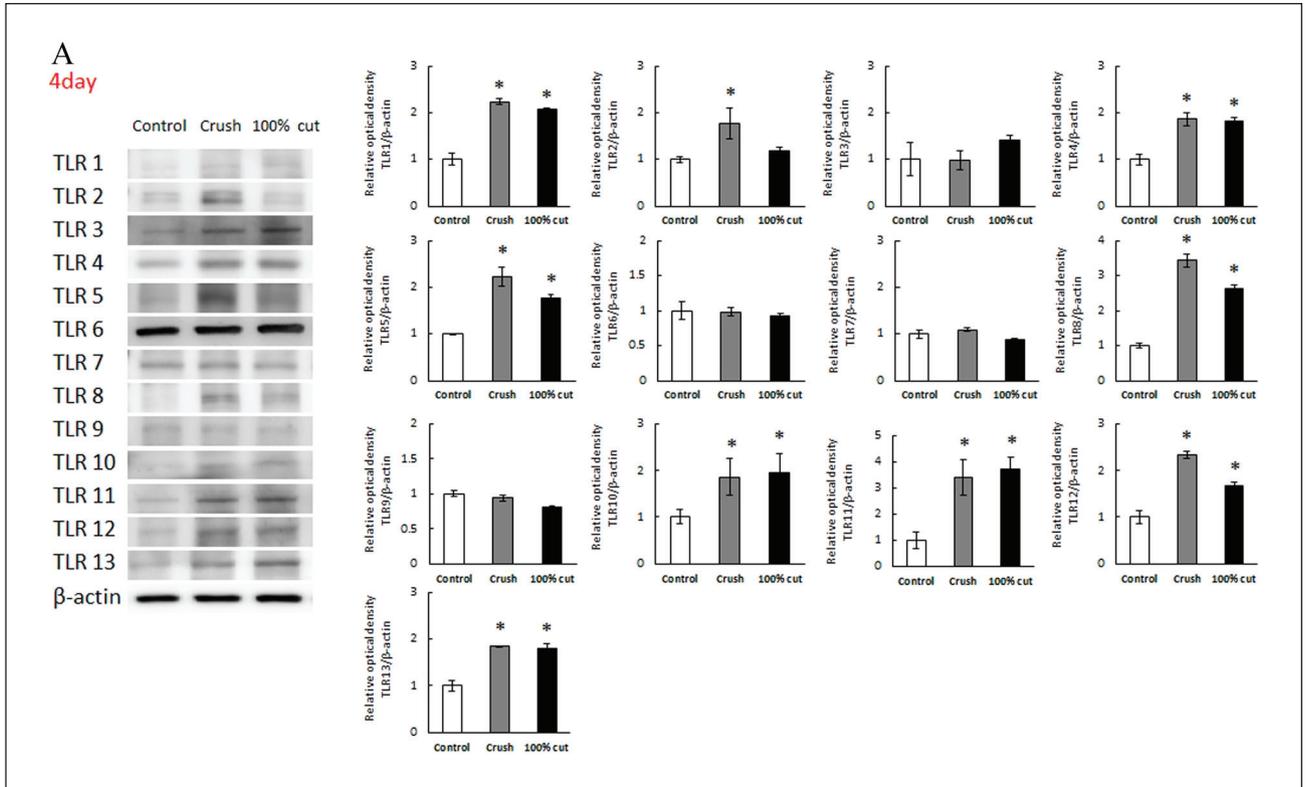


Figure 2. (Continued)

(Continued)

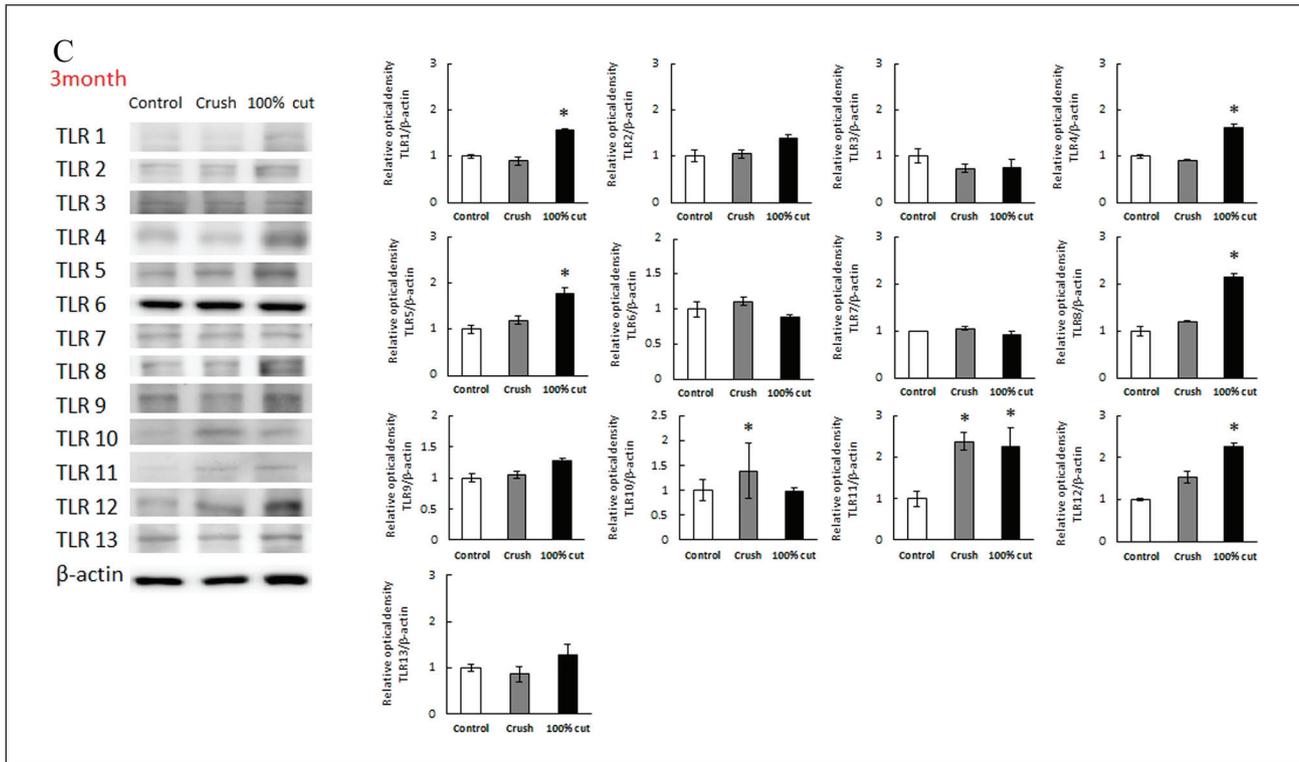


Figure 2. Western blotting results. Expression of TLR 1–13 proteins (A) 4 days, (B) 14 days, and (C) 3 months after crushing and cutting facial nerve injuries and in matched control rats. TLR: Toll-like receptor. * $p < 0.05$.

nerve. Negative regulators that inhibit myelination include the transcription factors c-Jun, Notch, Sox-2, and Pax-3 and the transcription control factor Id2. Negative regulators are mainly present in damaged nerves and promote deformation, creating an environment that promotes nerve survival and axonal regeneration.^{11–14} Besides these factors, TLRs, which are involved in innate immunity, have been found to be involved in Wallerian degeneration. Schwann cells and macrophages play important roles in Wallerian degeneration, resulting in nerve regeneration after peripheral nerve damage. By-products of axonal degeneration after nerve damage activate the expression of TLRs 2, 3, and 4, myeloid differentiation primary response 88 (MyD88) in Schwann cells and activate tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), and monocyte chemoattractant protein-1 (MCP-1). MCP-1 stimulates phospholipase A2 (PLA2), which in turn stimulates lysophosphatidylcholine (LPC), and Schwann cells directly degrade myelin. In addition, LPC expresses macrophage inflammatory protein-1 α (MIP-1 α), which promotes IL-1 β

secretion by Schwann cells and damages peripheral nerves within 3 days. IL-1 β , MCP-1, and MIP-1 α are secreted by Schwann cells of the peripheral nerves on days three to seven after nerve damage activates macrophages, and myelin is removed by phagocytosis.^{15–18} Although TLRs 2, 3, and 4 are associated with the intracellular signaling pathways of Schwann cells, the present study found that all TLRs were expressed after nerve damage. Although signaling pathways for all TLRs have not been identified, TLRs other than TLRs 2, 3, and 4 were shown to be indirectly involved in nerve damage and regeneration.

In this study, western blotting showed that the levels of TLRs 11 and 13 proteins increased at 14 days after crushing injury, while TLRs 10 and 11 increased at 3 months, accompanied by improvements in facial motor function. Moreover, the levels of TLRs 1, 2, 3, 4, 5, 8, 10, 11, 12, and 13 proteins increased at 14 days, and those of TLRs 1, 4, 5, 8, 11, and 12 increased at 3 months after the injury. These findings showed that the increase in TLR expression was dependent on the type of injury and

the time after nerve injury. In addition, facial nerve damage was more severe after a cutting than crushing injury, accompanied by the expression of additional TLRs. The difference in the types of TLRs expressed is thought to be due to the difference in histological changes after injury. According to a study using the sciatic nerve of rats, the histological characteristics of the distal nerve vary according to the type of injury and the time after the injury. In the case of crushing injury, nerves that regenerate over time after injury are observed. However, in the case of cutting injury, only completely degenerated nerves are observed at the distal part of the injury.^{14,19} Based on this, we hypothesized that TLRs 10, 11, and 13, which are significantly increased after crushing injury, are predominantly involved in the nerve regeneration process, and TLRs 1, 4, 5, 8, and 12, which are significantly increased after cutting injury, are involved in the degeneration process.

In particular, the level of TLR 4 increased in both the cutting and crushing groups at 4 days after nerve injury and only in the cutting group at 14 days and 3 months. This suggests that TLR 4 might be involved in nerve degeneration. It has been reported that TLR 4 is expressed in the cortical neuronal cells of mice and causes neuronal cell death in response to stimulation of brain ischemia. This is because TLR 4 expression in mice is associated with the activation of the proapoptotic signaling pathway involving Jun N-terminal kinase and the transcription factor AP-1. TLR 4 also promotes clearance of degenerating myelin and synaptic loss after nerve injury in the peripheral nerve. Activation of TLR 4 resulted in a delay in functional recovery after sciatic nerve injury in mice.^{20–22} TLR 1 showed an expression pattern similar to that of TLR 4 in our study. According to a study that analyzed TLR 1–9 expression in Schwann cells from mice, all TLRs 1–9 were expressed, but TLR 1 expression was especially increased after nerve injury. These results suggest that TLR 1 is expected to play an important role in the function of Schwann cells under stressful conditions such as nerve damage.²³ Similarly, TLRs 5 and 8, which also show comparable expression patterns, have been explored for regulating the process of neuropathic pain after nerve injury. TLRs 5 and 8 regulate neuroinflammation to increase the production of inflammatory mediators, resulting in nerve hyperexcitability. This process contributes to neuropathic pain after nerve injury.^{24–26}

It has been previously reported that unilateral sciatic nerve lesions lead to bilateral increases in TLR 9 mRNA and protein levels in the lumbar spine and the distant cervical dorsal root ganglia.²⁷ TLR 9 was also analyzed in our study, but no significant results were found. In our study, the facial nerve, a motor neuron, was used, and sensory and motor neurons exhibit differences in signaling and transcriptional pathways.²⁸ For example, the axotomy of sensory neurons leads to increased expression of transcription factors such as ATF-3, c-jun, Sox11, and STAT3, and regulators of translation such as arginase-1. The level of ATF-3 is sustained in motor neurons, resulting in stunted regeneration but only transient in sensory neurons, which is favorable for regeneration.²⁹ The underlying mechanism of differences in the expression of TLR 9 is still unknown. However, differences in the signaling and transcriptional pathways between sensory and motor neurons could be related. Few studies have been conducted on the role of TLRs 10–13 in the nervous system, especially TLRs 11–13, as these are receptors that are not found in humans. Several studies have demonstrated that TLRs 11–13 are expressed in astrocytes, microglia, and neuronal cells of the central nervous system of mice, which are increased during parasite infection. The activation of neurons, astrocytes, and endothelial cells of blood vessels through TLRs 11–13 could enhance their neuroprotective functions.^{30,31} In our study, the levels of TLRs 10 and 13 increased in both the cutting and crushing groups at 4 days after nerve injury and only in the cutting group at 14 days and 3 months. TLR 11 continuously increases after injury, regardless of the type or timing of the injury. This suggests that TLRs 10, 11, and 13 are involved in the regeneration process after nerve injury.

While the neural distribution of facial nerves is similar in humans, rodents, and lepidoptera, rats and rabbits have been frequently used as models to study human facial nerve function and regeneration. Electrophysiological examinations were performed to measure the degree of facial palsy and degree of recovery of the facial nerve after injury. In particular, in humans, the degree of palsy is measured using methods such as electroneurography, electromyography, nerve excitability tests, and maximum stimulation tests to evaluate the need for surgical intervention and to predict prognosis. In this study, the degree of facial palsy and

degree of recovery were determined by measuring the whisker movement of the vibrissae muscle and blink reflex of the eyelid rather than by neurological examination, thus eliminating the need to anesthetize rats several times. The present study found that whisker movement of the vibrissae muscle and blink reflex of the eyelids showed greater improvements over time after crushing than after cutting. The cutting group did not show complete paralysis in the behavioral test results. It is possible that synkinesis may occur during the process of regeneration of the facial nerve, resulting in whisker movement or an eyelid reflex. Another reason may be that, in addition to the facial nerve, the infraorbital branch of the trigeminal nerve is involved in autonomic innervation of the facial skeletal muscle during nerve regeneration. For the above reasons, facial muscle movements may appear in the behavioral test after cutting injury of the facial nerve.^{9,10,32}

This study has several limitations. First, we did not compare histological differences according to the method of injury and time after injury. In addition, since TLR was extracted from the entire facial nerve, it was not possible to analyze in which cell each TLR increased and in which pathway it acted. Third, we did not perform calculations to determine the sample size. Lastly, we did not perform morphological visualization by immunohistochemistry other than western blotting. However, this is the first report to demonstrate the expression of all TLRs in the facial nerve after injury. It also analyzes the expression of all TLRs, and in particular, the level of the expressed protein, not the mRNA level. Furthermore, long-term follow-up observations for up to 3 months were performed to clarify the differences according to the neurodegeneration and regeneration processes.

Conclusions

Changes in TLR expression were observed in the peripheral nerve region after facial nerve injury. TLRs 1, 4, 5, 8, and 12 are increased after cutting injury, which means that they are related to the nerve degeneration process. TLRs 10, 11, and 13 are increased after crushing injury, which means that they are related to recovery from facial palsy. These findings indicate that TLRs are involved in nerve degeneration and regeneration after facial nerve damage.

Declaration of conflicting interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics approval

The study protocol was approved by the Kyung Hee University Clinical Research Ethics Committee (KHMC-IACUC 21-005).

Animal welfare

The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation.

ORCID iD

Seung G Yeo  <https://orcid.org/0000-0001-8021-1024>

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