Increased H19 Long Non-coding RNA Expression in Schwann Cells in Peripheral Neuropathic Pain

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Background: Neuropathic pain is an intractable chronic pain condition caused by damage to the somatosensory system. Although non-coding RNAs such as microRNAs are important regulators of neuropathic pain, the role of long non-coding RNAs (lncRNAs) is poorly understood.

Methods: This study used a rat model of neuropathic pain induced by lumbar fifth spinal nerve ligation (SNL). Microarray analysis of lncRNAs in the lumbar fifth dorsal root ganglion was performed at day 14 after SNL. Expression levels of H19 were examined by using quantitative PCR. *In situ* hybridization was used to determine the distribution of H19 at day 14 after SNL. Schwann cells were isolated from peripheral nerves at day 14 after SNL.

Results: H19 lncRNA was greatly increased in the L5 dorsal root ganglion at day 14 after SNL and was significantly higher at and after day 4. In the dorsal root ganglion, H19 was detected mainly in nonneuronal cells but not in primary sensory neurons. Consistent with this, H19 expression was upregulated in Schwann cells isolated from peripheral nerves after SNL.

Conclusion: Increased H19 lncRNA in Schwann cells might be involved in neuropathic pain.
(J Nippon Med Sch 2019; 86: 215−221)

Key words: dorsal root ganglion, long non-coding RNA, neuropathic pain, primary afferent, Schwann cell

Introduction

Damage to the peripheral nerves of primary sensory neurons is a major cause of intractable neuropathic pain[1,2]. Although recent analgesics have improved neuropathic pain therapy, pharmacotherapy for neuropathic pain remains unsatisfactory because of its low efficacy and the risk of severe adverse effects[3]. After peripheral nerve injury, primary sensory neurons become sensitized and spontaneously transmit nociceptive information to the spinal cord. In addition, Schwann cells near the injury site undergo phenotypic changes that cause neuropathic pain. These cells induce neuroinflammation and sensitization of sensory neurons through multiple extracellular signaling molecules, such as cytokines and neurotrophic factors[4,5].

Non-coding RNAs have critical functions in physiological and pathological nociceptive processing. MicroRNAs, which repress target gene expression at the posttranscriptional level[6], are involved in pain modulation[7,8]. However, the involvement of long non-coding RNAs (lncRNAs), i.e., those longer than 200 nucleotides, is poorly understood in pain disorders, although tens of thousands of lncRNA genes are believed to be present in mammals[9]. LncRNAs were reported to have diverse physiological and pathological functions related to development and oncogenesis[10–13]. Regarding neuropathic pain,

Zhao et al. reported that lncRNA Kcna2 antisense RNA has a causal role through Kcna2 protein downregulation, which induces neuropathic pain in dorsal root ganglion (DRG) neurons[14].

lncRNA H19 was one of the first lncRNAs identified[15] and is an oncogene in a variety of cancers[16]. H19 is reported to function as a competing endogenous RNA[17] that binds to and blocks microRNAs through base pairing. In addition, H19 binds to protein molecules, to guide their localization[18]. Interestingly, H19 also functions as a precursor for microRNA miR-675, which is encoded in the first exon of H19[19]. In this study, we show that H19 is persistently increased in Schwann cells along the peripheral axon of primary sensory neurons under neuropathic pain conditions. H19 may thus be involved in the pathogenesis of neuropathic pain.

Materials and Methods

Animal Models

Male Sprague-Dawley rats (age 5-6 weeks) were used for all experiments. All experimental procedures were reviewed by the Animal Experiments Ethical Review Committee and approved by the President of Nippon Medical School (approval number, 27-037). All surgeries were performed under deep anesthesia with isoflurane inhalation (2%-3%). Neuropathic pain was induced by spinal nerve ligation (SNL), as previously described[20]. Briefly, the left lumbar fifth (L5) spinal nerve was exposed and tightly ligated with 4-0 silk thread at two sites separated by about 1 mm. For sham surgery, the L5 spinal nerve was exposed but not ligated. The L5 DRG, ventral root, dorsal root, and peripheral nerves proximal and distal to the ligated site were removed, frozen in liquid nitrogen, and stored at −80°C until RNA purification. The proximal peripheral nerve was collected between the DRG and ligated sites. For the distal peripheral nerve, a 10-mm length of peripheral nerve was collected distal to the ligated site.

Behavioral Tests

Hindpaw withdrawal response to mechanical stimulus, a measure of mechanical allodynia, was determined with von Frey filaments (Muromachi Kikai, Tokyo, Japan). The weakest force (g) that induced hindpaw withdrawal was regarded as the paw withdrawal threshold, when the rat responded at least three times in five trials. To examine thermal hyperalgesia, we used the Plantar test (Ugo Basile, Comerio, Italy). Each rat was placed on a glass plate, and a radiant heat generator below the plate was used to stimulate the rat twice, with an interval of at least 5 min. Paw withdrawal latency was defined as the mean of the two trial results.

Microarray Analysis

RNAiso Plus (Takara Bio, Shiga, Japan) was used to extract total RNA from L5 DRGs 14 days after SNL or sham surgery. LncRNA microarray analysis was performed by using a custom microarray slide that included transcripts catalogued as genes that are not known mRNAs in the SurePrint G3 Rat GE 8×60 K microarray slide (Agilent Technologies, Santa Clara, CA, USA) and rat transcripts that were potentially homologous to known human lncRNAs. Gene-specific probes were constructed in accordance with the manufacturer's protocol (Agilent Technologies). Total RNA (100 ng) was subjected to cyanine 3-labeled cDNA synthesis by using a Low Input Quick Amp WT Labeling Kit (Agilent Technologies). cDNA was hybridized on a microarray slide at 65°C for 17 hours. The hybridized slide was scanned by a DNA Microarray Scanner (Agilent Technologies), and fluorescent intensity was quantified with Feature Extraction software (Agilent Technologies). The data were analyzed with Gene Spring GX software (Agilent Technologies).

Quantitative Reverse Transcription-PCR

Total RNA (300 ng for DRG and the peripheral nerve; 100 ng for the ventral and dorsal roots) was reverse-transcribed with a random primer by using an iScript Select cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The purity and concentration of the extracted RNAs were measured with a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR was performed by using Power SYBR® Green PCR Master Mix and the StepOnePlus™ Real-time PCR System (Thermo Fisher Scientific). A primer pair for H19 was designed by using Primer-BLAST (https://www.nih.gov/) with the following sequences: forward, 5′-AGACATGACATGGTCCGGTG-3′, reverse, 5′-GGTGTTCAGGAAGGCTGGAT-3′. All samples were measured in triplicate. Amplification efficiency was calculated by assaying serially diluted samples, and relative expression levels were calculated.

In situ Hybridization

To produce an *in situ* hybridization probe for H19, a fragment of H19 was amplified from rat DRG-derived cDNA by using forward (5′-GCCAGTCAAGACTGAGGCTG-3′) and reverse (5′-GGGTTCAAGGTAGGGGGAAG-3′) primers attached with *Eco*RI and *Bam*HI restriction sites, respectively, and then inserted into pBluescript II (Agilent Technologies). After linearization, a digoxigenin-labeled RNA probe was synthesized by using T3 polym-
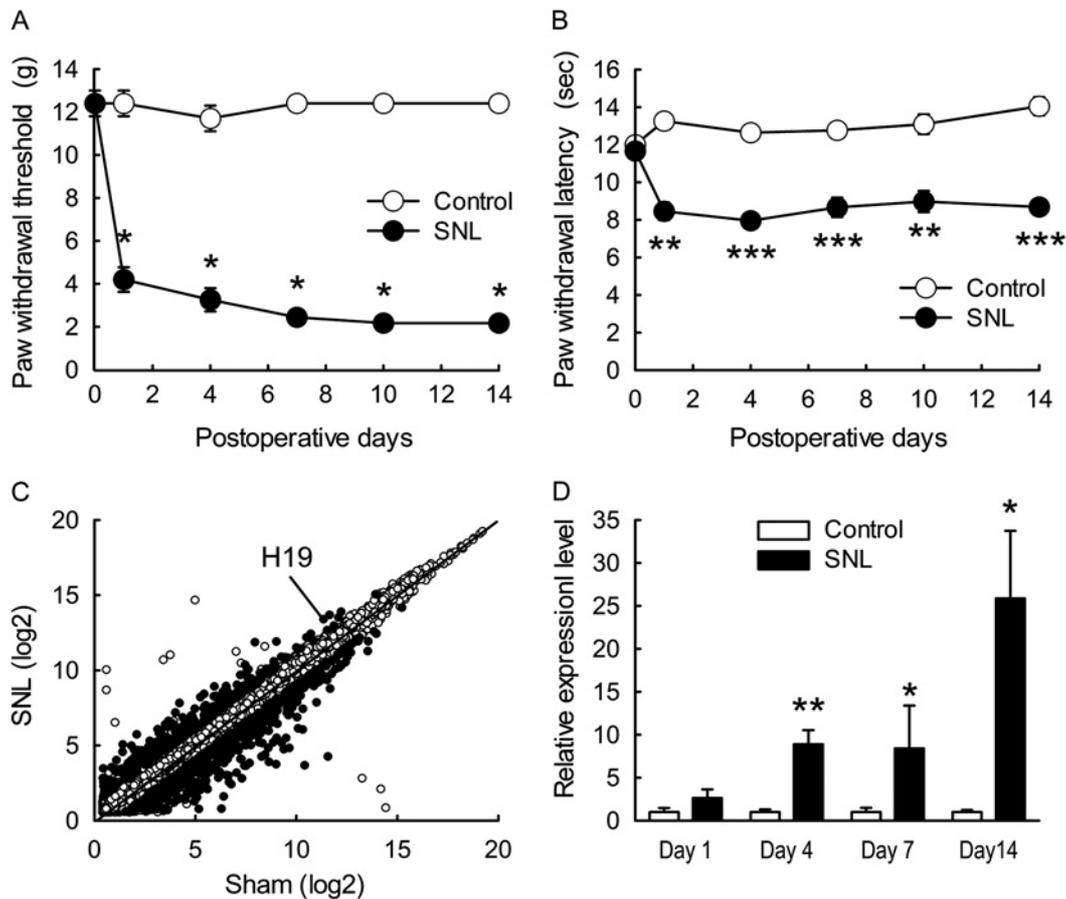
Fig. 1 Dynamic changes in H19 expression in a neuropathic pain model.
Mechanical allodynia (A) and thermal hyperalgesia (B) were examined on the control and SNL sides of rats after SNL ($n = 7$; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Mann-Whitney U-test for mechanical hyperalgesia and paired t-test for thermal hyperalgesia). (C) Microarray analysis was performed in the L5 DRG on the control and SNL sides 14 days after SNL. Differentially expressed genes are shown by filled circles. (D) Time course of changes in H19 expression was examined in L5 DRG by using quantitative PCR ($n = 5$-6; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by paired t-test or Mann-Whitney U-test).

erase (Roche Diagnostics, Basel, Switzerland). For a control experiment, a sense probe was synthesized with SP6 polymerase.

Rats were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. DRG and peripheral nerve proximal to the ligated site were excised, post-fixed in the same fixative overnight at 4°C, and cryoprotected in 20% sucrose in PBS overnight at 4°C. The tissues were rapidly frozen in dry ice/acetone and sectioned at a thickness of 10 μm with a cryostat (Leica Microsystems, Wetzlar, Germany). The sections were treated with 10 μg/μL proteinase K for 5 min. After incubation in 4% paraformaldehyde/PBS for 20 min, the sections were hybridized with an RNA probe in hybridization buffer (50% formamide, 5× SSC pH 4.5, 1% SDS, 50 μg/mL heparin sodium, and 50 μg/mL yeast RNA) at 65°C overnight. The sections were washed with wash buffer (50% formamide, 5× SSC pH 4.5, and 1%

SDS) at 65°C for 30 min and then washed thrice with a second wash buffer (50% formamide and 2× SSC pH 4.5) at 65°C for 30 min. Then, an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2,000; Roche Diagnostics) was incubated at 4°C overnight. The sections were stained with BM-purple (Roche Diagnostics) for 5 days. Images were captured by using a high-resolution microscope equipped with a computer (Olympus, Tokyo, Japan).

Schwann Cell Isolation

The bilateral peripheral nerves (from the L5 spinal nerve to the bifurcation of the tibial nerve and common fibular nerve) were excised, cut to a length of 1 mm, and preserved in F12 medium at 4°C. Nerves were treated with 5 mg/mL collagenase (Wako, Osaka, Japan) and 1 mg/mL dispase II (Roche Diagnostics) in PBS for 30 min at room temperature. After trituration with gentle pipetting, the cell suspension was passed through a cell
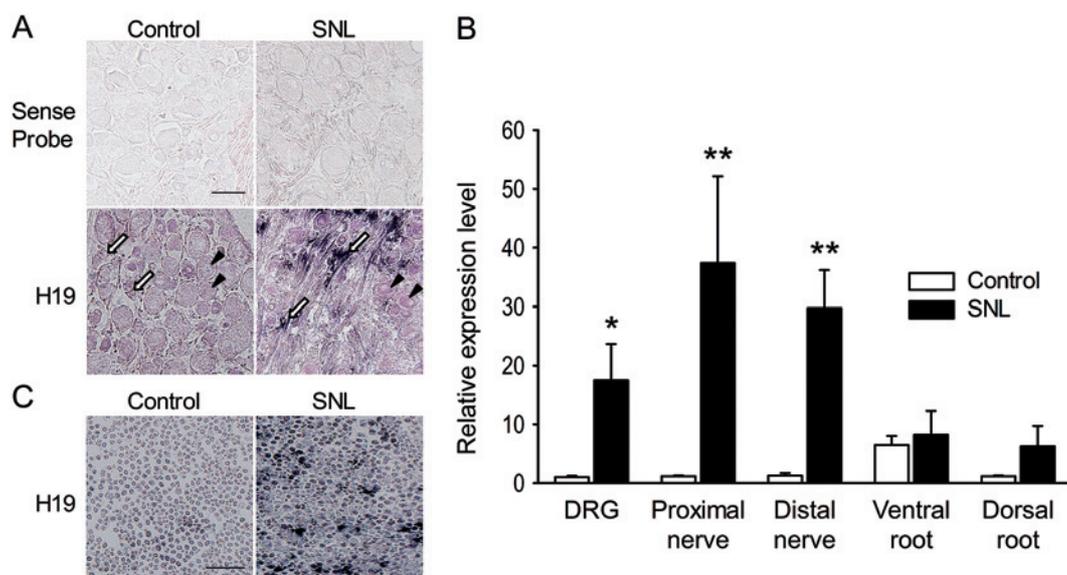
Fig. 2 Upregulation of H19 expression along the peripheral nerve of primary sensory neurons. (A, C) H19 distribution was examined in the L5 DRG (A) and proximal nerve (C) of control and SNL sides 14 days after SNL by using *in situ* hybridization with a sense or antisense probe for H19. Arrowheads and arrows indicate primary sensory neurons and non-neuronal cells, respectively. Scale bars represent 100 μm. (B) H19 expression levels in different nerve regions were examined 14 days after SNL by using quantitative PCR (n = 5-9; *P < 0.05 and **P <0.01 by paired *t*-test).

strainer (pore size 60 μm, pluriSelect, Life Science, Leipzig, Germany) to exclude debris, and the cells were seeded on culture plate for 1 hour at 37°C under 5% CO_2. Adherent cells were collected by using 0.05% trypsin/EDTA (Wako) and treated with Thy1.1 monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) and rabbit complement (Sigma-Aldrich) to remove fibroblasts, as previously described[21,22]. The resultant Schwann cells were confirmed by immunostaining against a specific marker, S100 (Sigma-Aldrich).

Statistical Analysis

Values are expressed as the mean ± SEM. SPSS software (IBM) was used for statistical analyses. Normality of data was assessed by the Shapiro-Wilk test. If a normal distribution was assumed, the paired t-test was performed. If a normal distribution was not assumed, the Mann-Whitney *U*-test was performed. All tests were two-tailed, and a *P* value of <0.05 was considered statistically significant.

Results

H19 was Persistently Upregulated in DRGs Under Neuropathic Pain Conditions

SNL was performed in rats to induce neuropathic pain. After SNL, the paw withdrawal threshold and latency were significantly decreased in response to mechanical and thermal stimulus, respectively, for at least 14 days

(**Fig. 1A and B**), which indicated that the rats had developed mechanical allodynia and thermal hyperalgesia. Microarray analysis to identify lncRNAs potentially involved in neuropathic pain revealed many lncRNA candidates with significant changes in expression in the L5 DRG at day 14 after SNL (**Fig. 1C**). Of these, we focused on the well-characterized lncRNA H19, which showed the greatest increase among the abundant lncRNA candidates. Quantitative PCR revealed that H19 expression in the DRG increased during the 14-day period after SNL (**Fig. 1D**).

H19 was Upregulated Along the Peripheral Nerve of Primary Sensory Neurons

To identify the cell type that expressed H19, *in situ* hybridization for H19 was performed. In the L5 DRG 14 days after SNL, H19 expression was observed in non-neuronal cells around nerve fibers but was much lower in primary sensory neurons (**Fig. 2A**). The sense probe for H19 yielded no signal (**Fig. 2A**). Therefore, we examined H19 distribution along nerve fibers. In peripheral nerves proximal and distal to the ligated site, H19 was markedly upregulated at day 14 after SNL (**Fig. 2B**). Consistent with this, *in situ* hybridization showed that H19 in non-neuronal cells was increased in proximal peripheral nerve at day 14 after SNL (**Fig. 2C**). In contrast, H19 expression was unaffected in the central nerve of DRG neurons (dorsal root) and motor nerve (ventral
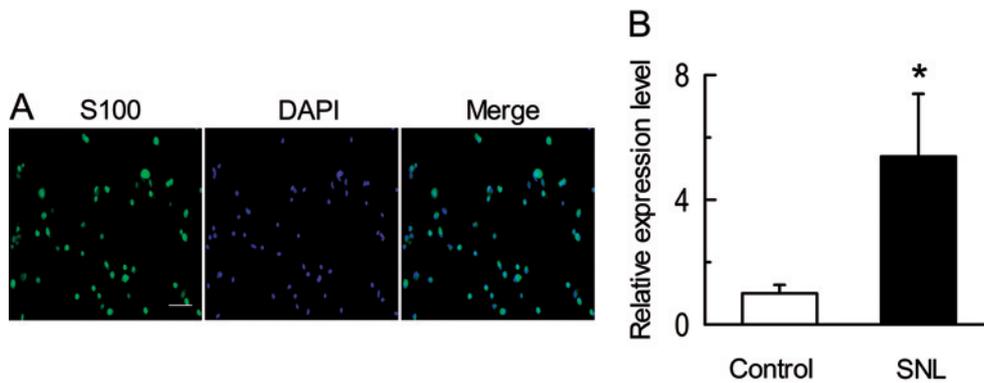
Fig. 3   H19 expression in Schwann cells after SNL.
(A) Schwann cells isolated from peripheral nerves were confirmed by S100 immunostaining and DAPI staining. The scale bar represents 100 μm. (B) H19 expression was examined in Schwann cells isolated from peripheral nerves proximal and distal to ligated sites 14 days after SNL ($n = 8$; *$P < 0.05$ by Mann-Whitney U-test).

root), although their basal expression levels were higher than those in the DRG and peripheral nerve (**Fig. 2B**).

H19 was Upregulated in Schwann Cells Under Neuropathic Pain Conditions

To determine whether H19 expression was increased in Schwann cells under neuropathic pain conditions, we isolated Schwann cells from the peripheral nerve. S100 immunostaining confirmed that complement-mediated cell lysis yielded Schwann cells at a high purity (>95%; **Fig. 3A**). H19 expression was significantly increased in Schwann cells isolated from peripheral nerves at day 14 after SNL (**Fig. 3B**).

Discussion

This study showed that H19 lncRNA was greatly and persistently upregulated under neuropathic pain conditions. Moreover, H19 was increased in peripheral nerves proximal and distal to the injured site but not in afferent dorsal and efferent ventral roots. Schwann cells isolated from the peripheral nerve showed increased H19 expression after SNL, suggesting that H19 is mainly increased in Schwann cells along the peripheral nerve of primary sensory neurons. Therefore, H19 was increased in Schwann cells along the peripheral nerve of primary sensory neurons.

H19 expression was increased in the proximal and distal peripheral nerve regions, which indicates that such expression was not correlated with Wallerian degeneration in the distal nerve, where Schwann cells undergo well-characterized phenotypic changes to promote nerve regeneration. However, phenotypic changes in Schwann cells were also induced in the proximal nerve[23]. After sciatic nerve transection, a population of Schwann cells rapidly expanded in the proximal nerve and underwent proliferation[24]. Changes in expressions of Notch signaling molecules were induced in Schwann cells in the proximal nerve[25]. Therefore, H19 may be involved in the phenotypic change observed in Schwann cells in the proximal and distal nerves. However, the mechanisms underlying H19 upregulation remain unknown, although hypoxia induced H19 expression in glioblastoma[26]. Because H19 expression was unchanged in the motor axon (ventral root), the peripheral axon of primary sensory neurons may contribute to upregulation of H19 expression.

H19 upregulation in Schwann cells might have a role in neuropathic pain and/or nerve regeneration, both of which were observed after peripheral nerve injury. Schwann cells mediated neuropathic pain by inducing neuroinflammation through various mechanisms[4,27], including secreting inflammatory cytokines such as IL-1β and IL-6, which are involved in neuropathic pain[5,28]. TRPA1 activation in Schwann cells mediated macrophage infiltration, oxidative stress, and neuropathic pain in mice after nerve injury[29]. Interestingly, oxidative stress induced H19 expression in cholangiocarcinoma cell lines[30]. Consistent with proinflammatory changes in Schwann cells upon nerve injury, H19 activated inflammatory processes, such as cytokine production, although the functional significance of H19 in Schwann cells is unclear. H19 promoted IL-6 production by inhibiting let-7a/7b, which represses IL-6 expression[30], and IL-22 production partly through miR-675 encoded by H19[31]. In a rat model of temporal lobe epilepsy, H19 contributed to hippocampal microglia and astrocyte activation, resulting in the release of IL-1β, IL-6, and TNF-α, possibly through STAT3 signaling[32].

Alternatively, Schwann cells may be involved in nerve repair by means of trophic support of neurons[33,34]. After nerve injury, Schwann cells de-differentiate and activate a repair program that includes upregulation of neurotrophic factors such as nerve growth factor, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, and neurotrophin-3[33]. These growth factors are well-characterized in the modulation of nociceptive primary sensory neurons[35]. Schwann cells also support axon regrowth and undergo remyelination[36-38].

In conclusion, we revealed a characteristic change in lncRNA H19 expression, which was persistently upregulated in Schwann cells along peripheral nerve proximal and distal to the injury site. Functional analyses of H19 in Schwann cells will provide further insights regarding the underlying mechanisms involved in neuropathic pain and/or nerve regeneration.

Conflict of Interest: The authors declare no conflict of interest.

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