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Peripheral nerve injury and sensitization underlie pain associated with oral cancer perineural invasion

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1. Introduction

Oral cancer is painful, impairing speech and swallowing [10]. Perineural invasion (PNI), defined by cancer invading in, around, or along the nerve, is associated with pain and poor prognosis in oral cancer [9; 62]. It is unclear whether PNI causes nerve damage, which can result in neuropathic pain that is more severe and difficult to treat than non-neuropathic pain (*i.e.*, nociceptive pain) [23; 71]. Oral cancer cells release nociceptive mediators that

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contribute to nociceptive pain [35; 67; 86; 87]; the neuropathic component resulting from PNI is rarely addressed.

Peripheral neuropathy presents as a combination of positive (*e.g.*, pain) and negative symptoms (*e.g.*, sensory loss), which vary across disease conditions [23; 76]. As pathophysiological mechanisms of pain cannot be examined in patients, an examination of pain phenotypes helps provide clues to the underlying mechanisms [41; 76]. A subset of oral cancer patients exhibits symptoms suggestive of peripheral neuropathy such as intractable pain, function impairment, numbness, and formication [5; 44]. As diagnosis of nerve injury requires anatomical examination [34] that is challenging in patients, we seek to gain mechanistic insight by examining pain phenotypes in patients.

Neuropathic pain caused by peripheral disorders is predominantly driven by primary afferent neurons including both myelinated A- and unmyelinated C-fibers [20; 23]. Altered electrophysiological properties, along with neurochemical and anatomical changes in sensory neurons, contribute to different pain phenotypes following nerve injury [20; 23; 41]. The impact of PNI on electrophysiological and neuroanatomical properties of primary afferent neurons remains unknown.

We hypothesize that peripheral nerve injury and hypersensitive primary afferent neurons contribute to PNI-induced pain in oral cancer. We compared pain in oral cancer patients with and without PNI evaluated with a validated oral cancer pain questionnaire [47] that quantifies two prominent features of neuropathic pain - spontaneous pain (*i.e.*, pain felt without obvious external stimuli) and mechanical allodynia (*i.e.*, pain resulting from a non-painful stimulus) [41; 50; 58]. We produced a model of PNI-induced pain and probed the peripheral mechanisms using *in vivo* intracellular recordings and electron microscopic analysis of nerve ultrastructure.

2. Methods

2.1. Patients

A pre-existing database composed of a cohort of 69 oral cancer patients referred to Bluestone Center for Clinical Research and Oral Cancer Center at New York University College of Dentistry from 2010–2017 was used for data analysis. All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee (NYU IRB #10-01261) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients provided written informed consent prior to inclusion in the study. The database contains information for each patient including age, sex, racial/ethnic identity (white, Hispanic, Africa-American, Asian), drinking/smoking history (previous, never, current), and pathological information including the presence (yes or no) of PNI, lymphovascular invasion, extracapsular spread, nodal status, tumor staging (T1-4), and tumor thickness/depth of invasion. Nodal status was classified as node positive (N+) or negative (N0) by pathological examination of the neck dissection specimen following surgery. Anatomical location of the oral cancer (Supplemental Figure 1) includes tongue (55.1%), mandibular gingiva (23.2%), maxillary gingiva (7.25%), floor of the mouth (7.25%), buccal mucosa

(5.8%), and retromolar trigone (1.4%). Comprehensive history was obtained on patients to rule out medical conditions or disorders that may alter their pain perception. Inclusion criteria included biopsy-proven oral cancer that required surgical resection and completion of the University of California San Francisco Oral Cancer Pain Questionnaire (UCSFOCPQ) [47] within six weeks prior to surgery. Patients were excluded if they had 1) a history of surgical, chemotherapeutic or radiation treatment for oral cancer, 2) a diagnosed psychiatric condition, and 3) a history of addiction to pain medications or recreational drugs.

The UCSFOCPQ is a validated instrument specifically tailored to oral cancer pain [24; 47]. Patients were asked to refrain from taking pain medications for 24 hours prior to completing the UCSFOCPQ. The UCSFOCPQ was administered to patients meeting the inclusion and exclusion criteria at their initial visit, before being prescribed analgesics for any orofacial pain and before any treatment. The UCSFOCPQ consists of eight questions on a visual analog scale of 0–100 mm; patients were instructed to place a vertical line along the scale to approximate their orofacial pain level [47]. The UCSFOCPQ differentiates spontaneous and mechanical allodynia and determines the quality of pain. Questions 1 through 6 focus on the intensity, sharpness (like knife), and aching or throbbing nature of oral cancer pain and whether the pain was spontaneous (when patients are not talking, eating, or drinking) or function-related (when patients are talking, eating, or drinking). Question 7 was designed to determine the degree of pain sensitivity (like sunburn or raw skin) to touch. Question 8 addressed the level of functional restriction as a result of orofacial pain.

2.2. Oral cancer cell culture

Human oral squamous cell carcinoma (SCC) cell line (HSC-3) was purchased from ATCC. Cell passages between 4 and 10 were used for experiments. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, l-glutamine and sodium pyruvate (Gibco, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin sulfate, and 100 U/mL penicillin G at 37°C in 5% CO₂. Before cancer cell inoculation, HSC-3 cells were rinsed with phosphate-buffered saline (PBS) (no Ca² and no Mg²), detached with 0.25% trypsin-EDTA, centrifuged for 4 minutes at 1000 rpm, and resuspended in DMEM to a final concentration of 15,000 cells/µL. The cell line was authenticated by the ATCC human STR Profiling Cell Authentication Service and tested for mycoplasma contamination (MycoAlert™, Lonza) prior to use.

2.3. Mouse model of sciatic nerve PNI

Six- to eight-week-old female NU/J athymic (*Foxn1^{nu}*) mice (stock number 002019, Jackson Laboratory) were housed in a temperature-controlled room on a 12:12 light/dark cycle (6 AM to 6 PM), with *ad libitum* access to food and water. Immunocompromised mice were used to generate the PNI model because they are permissive for the growth of human oral cancer cells. Female mice were used as we routinely used female mice for our study of oral cancer pain [84–88] and male mice occasionally exhibit aggressive behaviors that require individual housing. All procedures were approved by the New York University Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for the use of laboratory animals in research. A mouse sciatic nerve PNI model was created using a published protocol [27] with some modifications. NU/J mice

were anesthetized using 2% isoflurane. The skin on the injection site was cleaned with povidone-iodine and alcohol wipes. A 1-cm incision was made on the skin and the right sciatic nerve was exposed by gently separating the muscles of the gluteus maximus and biceps femoris. The nerve was freed from the surrounding tissue and lifted with a sterilized pin with a bifurcating fork. Under a stereo microscope, 3 μ L of HSC-3 cells mixed in PBS at a concentration of 15,000 cells/ μ L was injected using a 10- μ L Hamilton syringe connected with a 30G needle (BD PrecisionGlide Needle). 3 μ L of precancerous dysplastic oral keratinocytes (DOK, 15,000 cells/ μ L) or PBS were injected into the mouse sciatic nerves as controls. Since no difference in von Frey thresholds, paw guarding scores, or nerve gross anatomy was found between DOK and PBS injected mice (data not shown), only PBS was used as a sham group throughout the rest of the experiments. The formation of a bulge at the injection site was used as an indicator of successful injection. After gently removing the needle, the nerves were covered with the underlying muscles and the skin was closed with skin closure clips (Roboz Surgical Reflex 7). Mice were placed on a heating pad and returned to the home cage upon full recovery.

2.4. Behavior measurements

Mice were allowed to acclimate to the behavior room, the experimenter, and the measuring device for two weeks before baseline measurements were taken. The experimenters were blinded to the experimental groups for all behavioral tests.

2.4.1. Conditioned place preference (CPP) test—To test for ongoing spontaneous pain, we used the CPP test [22; 25; 46; 65; 74]. We used a selective μ -opioid agonist DAMGO (3 μ g/kg i.p.) to produce CPP in mice with sciatic nerve PNI [65]. We performed a single trial CPP protocol on PID 18–22. The three-chamber CPP apparatus consists of two conditioning chambers with distinct tactile, visual, and olfactory cues, connected by a smaller neutral chamber that was brightly lit. The visual cues were horizontal striped and dot wall papers. The tactile cues were smooth and rough flooring. The olfactory cues were strawberry and mint. White noise was played to block out any extraneous sounds. On the first day (PID 18, preconditioning) of the experiment, mice were introduced to the neutral chamber and allowed to explore all 3 chambers for 1 hour. Baseline time spent in the chambers was measured using ANY-maze tracking software (Braintree, Scientific, Braintree, MA, USA). Exclusion criteria for mice were spending <20% or >80% time in a chamber. On the second, third and fourth days (PID 19–21, conditioning), mice received i.p. injection of saline followed by confinement into the appropriate pairing chamber for one hour, following which they were returned to their home cage. Four hours later, mice received i.p. injection of DAMGO (3 μ g/kg, 50 μ L) followed by confinement into the opposite pairing chamber for one hour. No significant group preference for a specific chamber was noticed at the baseline. The chamber in which each mouse spent less time at the baseline (the less preferred chamber) was paired with DAMGO to minimize false-positive results [25; 74]. On the fifth day (PID 22, testing), mice were once again allowed to freely explore the apparatus for one hour. Time spent in the chamber and distance travelled was recorded by ANY-maze tracking software (Braintree). We used five animals per group.

2.4.2. Guarding behavior measurement—Mice were placed in a raised acrylic glass chamber with a mesh bottom and allowed to habituate for 15 min. The paw guarding behaviors were measured over two 5 min sessions with a 10 min interval [55]. Mice were assigned a score of 0–3 (0, the right hind paw is in normal contact with the floor; 1, the mouse does not bear full weight on foot and the weight tilts the left paw; 2, the mouse holds foot completely above mesh; 3, the right paw is licked). The average score for the 2 trials was determined for each mouse per behavioral day. The final score was determined by multiplying the time spent in each score (in seconds) by its respective score and dividing by the total observation time (600 seconds)[55].

2.4.3. Von Frey test—Mechanical allodynia was examined using von Frey filaments in a separate group of mice. Mice were placed individually in a plastic cage with a wire mesh floor. The mid-plantar right hind paw was stimulated with a series of von Frey fibers (bending forces: 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1 and 2 g) with logarithmic increments in stiffness (TouchTest®, North Coast Medical Inc.) using the “ascending stimulus” method [28; 57]. The von Frey fibers were held perpendicular to the testing surface and applied to the paw with sufficient force to cause buckling. A positive response was recorded if the paw was sharply withdrawn or if there was an immediate flinch upon removal of the fiber. The fibers were presented at least five seconds apart to allow resolution of previous stimuli. The threshold for each trial was determined as the von Frey hair that elicited a withdrawal response in 40% (two out of five) of applications [57]. Three repetitive trials were averaged as the threshold for each mouse at different time points.

2.4.4. Toe spreading reflex test—Motor function was accessed by lifting the tail of the mouse and examining the toe spreading reflex. If a mouse fully separated its toes, the response was scored as 4, spreading with four toes was scored 3, spreading with two toes was scored as 1, and no spreading was scored as 0 [51]. The toe spreading motor test is more sensitive than gait analysis for motor function assessment following sciatic nerve injury [51].

2.5. *In vivo* single neuron intracellular recordings

Three weeks (PID 21–25) after HSC-3 cell inoculation (N=4) or sham surgery (N=4), we performed intracellular recordings in mice under anesthesia as previously described [11; 14; 37]. The mouse trachea was intubated, and the lungs ventilated using pressure-controlled ventilation (Inspira PCV, Harvard Apparatus) with humidified oxygen. Heart rate and blood pressure were monitored throughout the test period as a guide to depth of anesthesia. Inspired end tidal isoflurane concentration was maintained at 2% throughout the study. Isoflurane might change neuronal excitability in a concentration dependent manner [59]. In our previous studies no obvious difference in active and passive electrophysiological properties was found in subtypes of primary afferent neurons in mice anesthetized with 2% isoflurane [37] *versus* ketamine/xylazine combination [14; 15]. A dorsal midline incision was made in the trunk skin and the L4 dorsal root ganglion (DRG) and adjacent spinal cord were exposed by laminectomy. The tissue was continuously superfused with oxygenated artificial cerebrospinal fluid (127.0 mM NaCl, 1.9 mM KCl, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 2.4 mM CaCl₂, 26.0 mM NaHCO₃, and 10.0 mM D-glucose). The temperature of the recording chamber was maintained at 37°C ± 0.2°C using an infusion pump (MPRE8;

Cell MicroControls). Pool temperature adjacent to the DRG was monitored with a thermocouple (IT-23; Physitemp). Rectal temperature (RET-3; Physitemp) was maintained at $34^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with radiant heat. The L4 DRG soma was impaled with borosilicate microelectrodes (80–250 M Ω) containing 1 M potassium acetate.

DRG neurons were classified according to published protocols [11-14; 37]. Once a stable intracellular recording was obtained at the soma, we applied gentle pressure using a fine-tipped brush to locate the peripheral receptive field (RF) with the aid of a zoom stereomicroscope. For cells that did not respond to the brush, we searched the skin with increasingly stiffer probes, ending with sharp-tipped watchmaker forceps. Calibrated von Frey filaments (Stoelting, Wood Dale, IL) applied by hand for 2 seconds per trial were used to determine mechanical threshold and peripheral dynamic response properties (*e.g.*, rapid or slow adaptation). The mechanical threshold is defined as the force generated by the minimum von Frey hair that produces neuronal activity. We confirmed adaptation rate using a suprathreshold probe mounted on a micromanipulator. Low threshold mechanoreceptors (LTMRs, *i.e.*, tactile afferents) and high threshold mechanoreceptors (HTMRs, *i.e.*, nociceptors) were identified by a combination of soma membrane properties, rate of adaptation, mechanical thresholds, and ability to encode mechanical intensity [14; 15; 37; 89]. LTMRs exhibit narrow uninflected spikes with short afterhyperpolarization (AHP), inability to encode mechanical intensity, and with low mechanical thresholds. HTMRs were distinguished by their ability to encode stimulus intensity, moderate to high mechanical thresholds, and broad spikes with long AHP. In addition, LTMRs and HTMRs were confirmed by the application of vibratory stimuli to the RF (tuning fork of 256 Hz; SKLAR Instruments, West Chester, PA); only LTMRs will respond to the 256 Hz vibration stimulus. Muscle spindles were identified by their location in the deep tissue and response to muscular stretch or alternating flexion/extension of the extremity. Mechanically sensitive primary afferent neurons were further characterized into A ($> 1.2\text{m/s}$) or C ($< 1.2\text{m/s}$) subtypes based on their conduction velocity (CV). We did not subdivide neurons into A β / δ groups given the known overlap in physiological and anatomical properties between A β and A δ fibers and the arbitrary nature of the CV cutoff value [15; 31]. CV was calculated by conduction distance divided by spike latency. Conduction distances were measured for each afferent on termination of the experiment by inserting a pin through the receptive field (marked with ink at the time of recording) and carefully measuring the distance to the DRG along the closest nerve. Spike latency was obtained by stimulating the receptive field at the skin surface using a bipolar electrode (0.5 Hz). Neurons that are mechanically silent (*i.e.*, with negative receptive field) were further characterized into the excitable and the unexcitable groups based on their capability to produce an action potential following injection of a depolarization current into the soma. The excitable neurons were subsequently categorized into S (slow)-type and F (fast)-type neurons based on the presence or absence of an inflection in the repolarizing phase of the action potential, respectively. Post-discharge hyperpolarization (PDH) was defined by a transient inactivated state and membrane hyperpolarization following activation with the presence of a mechanical stimulus. Action potentials generated independent of an external stimulus were considered spontaneous firing. Action potentials generated after the removal of a mechanical stimulus were considered afterdischarge. Neurons included for analysis satisfied the following

requirements: resting membrane potential more negative than -30 mV, action potentials with an amplitude > 30 mV and with the presence of AHP. Passive membrane properties indicative of poor impalement (*i.e.*, extremely low input resistance and/or time constant) were excluded. Direct current output from an Axoclamp 2B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA) was digitized and analyzed off-line using Spike2 software (CED, Cambridge, UK). Sampling rate for intracellular recordings was 21 kHz throughout (MicroPower1401; CED).

2.6. Electron Microscopy (EM)

2.6.1. Specimen preparation—On PID 25, mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4). Sciatic nerves were dissected and fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde and 2% paraformaldehyde for 2 hours. Fixation was performed using the reduced osmium impregnation (rOTO) method with modification to overcome the staining gradients of large-volume dense en-bloc staining [39; 79]. In brief, after washing with 0.1 M cacodylate buffer, the nerves were placed in cacodylate buffer containing 2% OsO₄ for 1.5 hour, followed by incubation in 1.5% potassium ferrocyanide for 1.5 hour. The tissue was rinsed in double-distilled H₂O (ddH₂O) before being placed in a filtered solution of 1% thiocarbohydrazide (TCH) in ddH₂O for 20 min at room temperature. Nerves were washed in ddH₂O before they were placed in 2% aqueous OsO₄ for an additional 40 minutes at room temperature. Following another wash with ddH₂O, the nerve tissues were transferred to 1% aqueous uranyl acetate at 4°C overnight. En bloc lead staining was performed to enhance membrane contrast as described previously [77]. Nerve tissues were then dehydrated in a series of ice-cold ethanol solutions, processed in dry acetone, and gradually equilibrated with Durcupan ACM Araldite embedding resin (Electron Microscopy Sciences) by incubating in 30% Durcupan/acetone for 4 h, 50% Durcupan/acetone overnight, and 100% Durcupan overnight. Finally, nerve samples were embedded in fresh 100% Durcupan and placed in a 60°C oven for 48 hours to allow Durcupan polymerization and completion of the embedding procedure.

2.6.2. Imaging—The processed sample was trimmed, semi-thin sections were cut at 1 μ m and stained with 1% toluidine blue to find the area of interest. Thin sections (70 nm) were cut, and mounted on slot grids. For EM imaging, Grids were loaded onto the Zeiss grid holder. Images were taken with a beam acceleration of 20.0 kV and a working distance of approximately 2.0 mm, capturing 6144 by 4608 pixel images with a pixel size of 100 nm to 200 nm. A Talos120C transmission electron microscopy with Gatan (4k x 4k) OneView Camera was used for high resolution EM imaging. Ultrathin sections (50-60 nm) were imaged at 1250x. C-fibers in Remak bundles (number of Remak bundles, axon diameters) and A-fiber abnormalities (fiber number, myelin infolding, protrusions, and thickness, axoplasmic abnormalities) were counted and analyzed. Nine images were obtained from three animals per group for data analysis.

2.7. H&E staining

On PID 25, mice were transcardially perfused with 4% paraformaldehyde. Sciatic nerves at the tumor or PBS-injected regions were dissected and fixed in 4% paraformaldehyde for 2 hours. Nerves were then sent to the NYU Experimental Pathology Core for routine paraffin

embedding. Three 5 μ m nerve sections spanning 50 μ m apart from each other were sampled for H&E staining and nerve width measurement.

2.8. Statistics

GraphPad Prism 8, OriginPro 7.5, and IBM SPSS v25.0 were used to perform the statistical analyses. Standard parametric (Student's t-test, one-way ANOVA, two-way ANOVA) or nonparametric (Mann-Whitney U-test, Kruskal-Wallis) tests were used, depending on normality. Chi-square or Fisher's exact test was used to determine the difference in cell distribution between groups. Results were presented as mean $\pm$ standard deviation (SD), mean $\pm$ standard error of the mean (SEM), or median (range), wherever appropriate. Significance level was set at * P <0.05, ** P <0.01, *** P <0.001

3. Results

3.1. Patients with PNI report increased spontaneous pain and mechanical allodynia,

Patients with PNI (PNI+) were identified from the routine post-surgery pathology reports (Figure 1A). PNI+ patients (N=25) reported significantly higher scores for spontaneous pain intensity (Q1), sharpness (Q3) but not aching (Q5) when not talking, eating, or drinking than PNI- patients (N=44). The PNI+ patients also reported significantly higher functional pain intensity (Q2), sharpness (Q4), aching (Q6), and functional restriction (Q8) when talking, eating, or drinking. Pain evoked by touch at the site of oral cancer is also significantly greater in PNI+ patients (Q7, Figure 1B). No sex difference was found in pain scores on any of the eight pain questions in PNI+ patients (Figure 1C). Analysis of demographic and pathological features found presence of PNI was associated with higher T stage (T1 vs. >T1) and depth of invasion (Table 1). PNI was not associated with age, sex, race, alcohol or smoking status, nodal status, lymphovascular invasion, or extracapsular invasion (Table 1). There was no difference in PNI incidence or pain scores between patients with tongue cancer *versus* cancers in other oral locations (Supplemental Figure 1).

3.2. PNI mice exhibit spontaneous nociception and mechanical allodynia.

We produced an *in vivo* PNI model by microinjecting a human oral SCC cell line (HSC-3) into the right sciatic nerve (Figure 2A-B). We measured spontaneous pain-like behavior using the CPP assay and by quantifying paw guarding. PNI mice (N=5) exhibited CPP to pain relief, spending more time in the DAMGO-paired chamber than the saline-paired chamber (P <0.05, Figure 2C). Sham mice (N=5) did not demonstrate CPP behavior to DAMGO (Figure 2C). Paw guarding scores were significantly increased in PNI mice compared to the sham mice at PID 13 (P <0.05) and PID 21 (P <0.05, Figure 2D). PNI mice also exhibited mechanical allodynia measured by the von Frey test; the paw withdrawal threshold decreased from the baseline level at PID3 through PID23 (Figure 2E). Sham mice had a transient decrease in von Frey withdrawal threshold from their baseline at PID3 (P <0.001) that was not evident at later time points (Figure 2E). The mechanical threshold decrease from the baseline at PID3 observed in both groups is likely due to the incision in the skin and nerve microinjection. Mechanical thresholds were significantly lower in PNI mice (N=8) than sham mice (N=7) at PID 8 and at later intervals (P <0.001, Figure 2E). Mild functional restriction of the paw in the PNI mice was observed at PID23 using the toe

spreading reflex assay (Supplemental Figure 2A). Locomotion activities measured using distance traveled did not differ between the two groups (Supplemental Figure 2B).

3.3. PNI induces altered electrophysiological properties of primary afferent neurons.

Cellular receptive field was widespread along the L4 dermatome innervating both hairy and glabrous skin (Figure 3A). We recorded 56 neurons from four PNI mice and 42 neurons from four sham mice; neurons were sub-classified based on their mechanical response properties, conduction velocities, and spike shapes (Figures 3B-E). Mechanosensitive nociceptors including both AHTMRs and CHTMRs were hypersensitive in PNI mice with significantly lower mechanical thresholds than those from sham mice (Figures 3D and 3F). All AHTMRs (5/5) recorded from PNI mice, but none from sham mice (0/6), lacked post-discharge hyperpolarization (PDH) during the presence of the mechanical stimuli and exhibited afterdischarge with removal of the stimuli (Figures 3D-E). In contrast, fast conducting tactile afferents, or LTMRs from PNI mice (N=6) exhibited mechanical desensitization, with increased thresholds compared to the sham mice (N=18, $P<0.05$, Figures 3D-F). In PNI mice, conduction velocities were significantly decreased in LTMRs and AHTMRs (Figure 3G), and a larger percentage of neurons were mechanically silent (*i.e.*, negative receptive field, 78.6%) compared to sham mice (37.2%, $P<0.001$, Figure 3E). In addition, a higher percentage of neurons were electrically unexcitable (*i.e.*, fail to generate action potentials following injection of a depolarization current into the soma, 23%) in PNI mice while no neurons in the sham group are unexcitable (0%, $P<0.001$, Figure 3E).

3.4. PNI induces nerve injury.

The nerves from the PNI mice are dilated (Figures 4A-C); the presence of the cancer cells within the nerve is visible from H&E stained cross sections of nerves. Compared to sham mice (N=3, Figures 4B-C), axons from PNI mice (N=3, Figures 4D-I) are disorganized and pathological features are widespread. The counts of myelinated fibers and Remak bundles that contain C-fibers are significantly reduced in PNI mice (Figure 4J). In the remaining myelinated fibers, both myelin and axon alterations are observed in PNI mice including: myelin invaginations and protrusions, myelin splitting, segregation, and thinning, myelin infoldings within Schwann cell cytoplasm, and accumulation of dark organelles within the axoplasm (Figures 4D-I). More nerves are abnormal in PNI mice (69.5%) compared to 11.7% in sham mice (Figure 4K). C-fibers within the Remak bundles of the PNI mice are dilated, with 37% increase in diameter compared to sham mice (Figures 4D-F and 4L).

4. Discussion

We report that oral cancer patients with PNI experience greater spontaneous pain and mechanical allodynia. In a mouse model of PNI, we provide neuroanatomical and electrophysiological evidence that pain associated with oral cancer PNI is neuropathic, driven by nerve injury and nociceptor sensitization.

Pain phenotype differences between patients with PNI and without PNI reflect differences in the underlying mechanisms. Oral cancer releases mediators such as NGF, TNF, BDNF, and

ATP, leading to nociceptive pain [22; 35; 48; 49; 67; 86; 87]. Nerve injury produced by PNI, further exacerbates pain. Higher pain severity in patients with PNI can be explained by the involvement of both nociceptive and neuropathic component, in contrast to nociceptive pain experienced by patients without PNI. Sharp pain is mediated by A-fibers, and aching/dull pain is mediated by C-fibers [56]. Oral cancer patients with PNI reported greater sharp pain, as well as aching pain evoked by oral function, suggesting increased sensitization of A- and C-fibers, respectively. Oral cancer patients with PNI also reported higher levels of spontaneous pain sharpness, but not spontaneous aching, suggesting potentially increased spontaneous discharges in A- but not C-fibers.

In a mouse model of PNI we demonstrated that in parallel to PNI-induced pain, A and C HTMRs are hypersensitive: both AHTMRs and CHTMRs exhibited reduced mechanical thresholds; AHTMR exhibited afterdischarge and loss of PDH. The hypersensitive state of nociceptive afferents is important for the induction and maintenance of activity dependent central sensitization [6]. Decreased mechanical thresholds in both A and C HTMRs are consistent with mechanical allodynia or increased touch pain sensitivity [12; 13; 29; 56; 76]. LTMRs, which have been proposed to drive touch-induced mechanical allodynia [20; 41; 56], are desensitized, in agreement with previous findings on other nerve injury models [12; 13; 37]. LTMR desensitization, together with decreased conduction velocity in A fibers, and increased percentage of mechanically insensitive and electrically unexcitable neurons in PNI mice, reflect compromised transduction (due to terminal atrophy) and conduction (due to loss of axons or myelin) following nerve damage [76]. LTMRs desensitization and increased percentage of mechanically insensitive neurons induced by PNI could also result in a decreased tactile input into the spinal cord, producing disinhibition of the nociceptive circuitry, ultimately enhancing nociceptive sensation [12; 36; 45]. In our model, spontaneous pain cannot be explained by spontaneous activities developed in injured A or C fibers as others proposed [17; 29; 30; 82], since we did not observe spontaneous activities in these fibers. Spontaneous pain induced by PNI, may be due to central sensitization [7] or spontaneous activities arise from primary afferent neurons of adjacent uninjured nerves [30; 80].

We provided anatomical evidence of nerve injury and Schwann cell abnormalities in mice with PNI. We showed that sciatic nerves with PNI have a significant reduction in the density (counts per area) of A and C axons. Sciatic nerves with PNI exhibited more degenerating myelinated fibers; myelin infoldings into both axons and Schwann cell cytoplasm were observed. Such infoldings have been reported in painful demyelinating conditions such as the Charcot-Marie-Tooth disease [16; 33; 60]. Schwann cells form myelin and ensheath C-fibers [18; 69]. During nerve injury, Schwann cells are activated and break down myelin, which can result in decreased conduction velocity and failed action potential generation [11; 13; 19; 75; 78]. Activated Schwann cells release glia mediators that generate neuropathic pain in non-malignant diseases [2; 8; 18; 19; 42; 78; 83]. Schwann cells and cancer cells reciprocally interact to promote cancer growth and invasion [3; 26; 63; 70; 73]. Schwann cell activation induced by cancer mediators could result in myelin breakdown. Cancer invasion and growth within the nerve breaks down the extracellular matrix and compresses the nerve, which could result in a physical damage to the nerve and a further injury response in Schwann cells. Supernatant collected from oral cancer activated Schwann cells evokes pain-

like behaviors in mice [63]. The data we reported here further support a potential involvement of Schwann cells in oral cancer PNI and associated pain.

The sciatic nerve PNI model we characterized here will provide a new approach to study the tumor-nerve interaction *in vivo* and help elucidate novel molecular targets underlying pain induced by oral cancer PNI. The molecular mechanisms of PNI-induced pain in oral cancer are poorly understood; consistent *in vivo* animal models are lacking [4; 40; 68]. Studying PNI is challenging as the occurrence of PNI varies in time, anatomical location, and involved nerve fibers; extensive histopathological examination of the resected tumor is required for PNI detection [27]. The two published models in head and neck cancer PNI include an *in vitro* co-culture model of isolated DRGs with oral cancer cells [40] and an *in vivo* co-culture model of isolated DRGs with oral cancer cells grown on chick chorioallantoic membranes [68]. These co-culture models do not allow nociceptive behavioral testing. Using these co-culture models and molecular analysis in human tissues, studies have shown that PNI is a selective process that requires activation of signaling pathways involving neurogenesis and regeneration, extracellular matrix remodeling, cell adhesion, wound healing, and chemotaxis [1; 4; 9; 64; 91]. Proposed molecular candidates mediating head and neck cancer PNI such as MMP2, galanin, PGE2, BDNF [32; 64; 91] are also known to regulate pain signaling; the contribution of these molecules in PNI-induced pain needs to be examined in future studies.

Additional experiments are needed to address some other important questions. We only used female mice in our study. We did not find a sex difference in PNI incidence or PNI associated pain in patients, consistent with findings from other studies in oral cancer patients with PNI [38; 90; 91]. In mixed populations of PNI+ and PNI- patients, the majority of studies found no sex difference in pain according to a systemic review [52], while others found oral cancer pain is more severe in women [61; 65; 66] as well as female mice with tongue cancer [65; 66]. The lack of sex-difference in pain associated with oral cancer PNI should be confirmed in a larger patient population and animal models. Future studies could incorporate other neuropathic symptoms such as numbness, paresthesia, and altered heat sensitivities to improve profiling for neuropathic pain in patients [23]. For the mouse model, heat behavioral testing and single fiber recordings in the A and C nociceptors [54] could be performed to understand the impact of PNI on heat sensitivity and the underlying peripheral mechanisms. Lastly, the peripheral and central neural substrates that drive spontaneous pain induced by oral cancer PNI need to be explored further in patients and animal models.

There are limitations to our study. False-negative reporting of PNI is a well-known issue in cancer pathology [21]. It is possible that PNI was under-reported in our patient cohort, which could diminish the pain difference between the groups. We used immunocompromised mice that lack mature T cells. The impact of T cells on pain has been reported in non-cancer pain models [43; 53; 69; 72]. The effect of T cells on oral cancer pain is unknown. Our PNI model can be developed with syngeneic cell lines and immunocompetent mouse strains in the future to compare with pain phenotypes of the current model. Lastly, the sciatic nerve is not an anatomically relevant location for oral cancer PNI; molecular and genetic differences exist between DRG and trigeminal ganglion (TG) neurons [81]. The nerves innervating the mouse oral cavity, however, are small and

difficult to access. The sciatic nerve PNI model produces reproducible tumor infiltration into the nerve, and is easy to visualize, access, and quantify [27].

We conclude that PNI-induced pain in oral cancer is neuropathic by nature. Patients with severe neuropathic pain have worse quality of life; their pain is more difficult to manage [71]. Pain resulting from cancer PNI is understudied. There is no treatment for PNI-induced pain. Our study provides a basis for the investigation of molecular and cellular mechanisms of PNI-induced pain and also for testing drug targets that can be used to treat PNI-induced pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Oral cancer patients with PNI experience greater pain based on the UCSFOCPQ. (A) A representative image of PNI from a resected human oral cancer specimen. Oral cancer cells (dark purple cells, yellow arrows) infiltrated the nerve. Yellow dashed line marks the boarder of a nerve. Scale bar=100 μ m. (B) Oral cancer patients with PNI experience increased pain intensity, sharpness when not eating, drinking, and talking compared to patients without PNI. Oral cancer patients with PNI experience increased pain intensity, sharpness, and aching when eating, drinking, and talking compared to patients without PNI. Oral cancer patients with PNI report higher levels of touch pain and function restriction. No difference in spontaneous aching is found between the two groups. (C) No difference in pain scores was found between female and male patients with PNI. Data were presented as mean $\pm$ SEM. Independent student t-test, * $P<0.05$, ** $P<0.01$.

Figure 2.

PNI Mice exhibited spontaneous nociception and mechanical allodynia. (A) PNI mice received 3 μ L PBS containing 4.5×10^4 HSC-3 (white arrow indicates the injection site) into the right sciatic nerve (*) by microinjection. Sham mice received 3 μ L PBS injected into the nerve. (B) A visible cancer developed in the mouse sciatic nerve at PID24. (C) PNI mice (N=5) showed a preference towards the DAMGO-conditioned chamber whereas sham mice (N=5) did not change their chamber preference. (D) The guarding score was significantly higher in PNI mice (N=5) than sham mice (N=5) at PID13 and PID21. (E) Both PNI (N=8) and sham mice (N=7) exhibited a decrease in von Frey paw withdrawal mechanical thresholds from the baseline at PID3. The mechanical threshold remained lower than the baseline in PNI mice throughout the observation period following cancer injection, while sham mice fully recovered to their baseline level at PID8. Mechanical thresholds were lower in PNI mice measured at PID8, 10, 14, 17 and 23. Data were presented as mean $\pm$ SEM. Two-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. Electrophysiological properties of primary afferent neurons affected by PNI. (A) Set up for *in vivo* intracellular (IC) recordings in the L4 DRG of a sciatic nerve. Green area represents HSC-3 cells inoculated into the sciatic nerve. Blue shaded area represents the L4 dermatome. Spinal vertebrae (grey area inside the oval) at the L4 regions were dissected open to expose the L4 DRG for electrode insertion and recordings. (B) Flow chart for DRG cell classification. Em: resting membrane potential. RF: receptive field. MT: mechanical threshold. F: fast. S: slow. UNEX: unexcitable. (C) Examples of distinct action potential shapes between LTMRs and AHTMRs. (D) Examples of typical evoked response by mechanical stimulation in LTMRs and AHTMRs recorded from L4 DRG of both sham and PNI animals. Top panels: LTMRs display a rapidly adapting (RA) response. Notice the mechanical threshold differences between LTMRs in sham and PNI mice. Bottom panels: Control AHTMRs exhibit a transient activation and membrane hyperpolarization by mechanical stimulation (*i.e.*, PDH), while the AHTMRs in the PNI group exhibit a sustained activation in the presence of the mechanical stimuli (loss of PDH). AHTMRs exhibit afterdischarge following removal of the mechanical stimuli. Mechanical threshold is lower in AHTMRs recorded from PNI mice. Scale bar: 0.3 sec, 20 mV. (E) Distributions of types of fibers recorded in PNI (N=4) and sham mice (N=4). P-RF: positive receptive field. N-RF: negative receptive field. MS: muscle spindles. UNEX: unexcitable. In sham animals, we recorded 18 LTMRs, 6 AHTMRs, 4 CHTMRs, 5 MS, 3 fast (F)-type, 6 slow (S)-type, and 0 unexcitable neurons. In PNI mice, we recorded 6 LTMRs, 5 AHTMRs, 3 CHTMRs, 2 MS, 17 F-type, 10 S-type, and 13 unexcitable neurons. In PNI mice, a greater percentage of neurons failed to generate action potentials (23%) compared to sham mice (0%, $P<0.001$) following a depolarizing current injection into the soma. A smaller percentage of neurons

were RF positive in PNI mice (37.2%) compared to sham mice (78.6%, $P<0.001$). (F) Mechanical thresholds (MT) of LTMRs were higher while those of AHTMRs and CHTMRs were lower in the PNI mice compared to the sham mice. (G) Conduction velocities (CV) of both LTMRs and AHTMRs were reduced in PNI compared to sham mice. Student's t-test or Mann-Whitney's U-test, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 4.

Sciatic nerve anatomy affected by PNI. Representative H&E stained images of nerves (x1250) from sham (A) and cancer (B) mice. Notice dark purple cancer cells within the nerve in (B). Scale bars: 50 μ m. (C) Tumor infiltrated nerve segments taken from PNI mice are larger in width compared to PBS-injected nerve segments from sham mice. N=3 per group. (D, E) Representative EM image of nerves from sham mice. (D) The sham animal group shows large numbers of unremarkable large and small myelinated axons. The axoplasm contains scattered organelles and the contours of the myelinated axons are circular with few myelin invaginations/foldings. Scale bar: 10 μ m. (E) The higher magnification insert of D shows numerous Remak bundles (arrowheads) containing unmyelinated axons, which are typically of a uniform diameter. No actively degenerating axons or tumor cells are identified. Scale bar: 5 μ m. (F-I) Representative EM images of nerves from PNI mice. (F) Myelin invaginations are found protruding into axons as excessive infolding (*). Intraxonal accumulation of dark organelles (arrowhead) often with a narrow myelin sheath are seen only in PNI mice. C-fibers within the Remak bundles (arrowhead) are larger than those shown in image D. Arrow: cancer cells. Scale bars: 10 μ m. (G) Myelin (arrow) shows evaginations into the Schwann cell cytoplasm. N: Schwann cell nucleus. Unmyelinated axons within Remak bundles (arrowhead) are larger in diameter. Scale bars: 5 μ m. (H) Subtle, perhaps artificial, myelin segregation (arrow). Scale bars: 4 μ m. (I) Myelin splitting (arrowhead) is occasionally seen in PNI nerves. There is an axon (arrow) containing large numbers of organelles and a thin myelin sheath which may reflect axonal dilatation with accumulated organelles and compensatory thinning of the myelin from nerves of PNI mice. Scale bars: 4 μ m. (J) Mice with sciatic nerve PNI have significantly reduced counts of myelinated axons and Remak bundles. Data are expressed as mean $\pm$ SEM per 2500 μ m². (K)

The percentage of abnormal A-fibers is significantly increased in PNI mice. (L) C-fiber diameters are larger in PNI mice. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

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Table 1.

Patient demographic and clinical characteristics.

	PNI (-)	PNI (+)	P
Age (years) mean $\pm$ STD (n)	64.5 $\pm$ 15.2 (44)	62.7 $\pm$ 13.5 (25)	0.63
Sex (n, %)			0.22
Male	25, 56.8%	10, 40.0%	
Female	19, 43.2%	15, 60.0%	
Race (n, %)			0.60
White	28, 63.6%	12, 48.0%	
Asian	7, 15.9%	6, 24.0%	
Hispanic	4, 9.1%	4, 16.0%	
African	5, 11.4%	3, 12.0%	
Smoking (n, %)			0.81
Previous	19, 43.2%	11, 44.0%	
Current	11, 25.0%	5, 20.0%	
Never	13, 29.5%	9, 36.0%	
N/A	1, 2.3%	0	
Alcohol drinking (n, %)			0.36
Previous	9, 20.5%	8, 32.0%	
Current	26, 59.1%	11, 44.0%	
Never	7, 15.9%	6, 24.0%	
N/A	2, 4.5%	0	
T status (n, %)			0.01 *
T1	23, 52%	5, 20.0%	
T1+	21, 48%	20, 80.0%	
Nodal status (n, %)			0.30
N0	15, 34.1%	13, 52.0%	
N+	23, 52.3%	11, 44.0%	
N/A	6, 13.6%	1, 4.0%	
Lymphovascular invasion (n, %)			0.23
Yes	8, 18.2%	8, 32.0%	
No	32, 72.7%	14, 56.0%	
N/A	4, 9.1%	3, 12.0%	
Extracapsular invasion (n, %)			1.0
Yes	7, 15.9%	5, 20.0%	
No	22, 50.0%	17, 68.0%	
N/A	15, 34.1%	3, 12.0%	

	PNI (–)	PNI (+)	<i>P</i>
Depth of invasion (mm) mean ± STD (n)	6.4±5.1 (31)	11.6±6.5 (16)	0.01 *

* indicates statistical significance between the groups.